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NUCLEOTIDE INTERMEDIATES IN THE BIOSYNTHESIS OF POLYSACCHARIDES

JACK L. STROMINGER*

A little over 10 years ago, Leloir and his collaborators (1) discovered a cofactor for galactose utilization in yeast, isolated the compound and showed that it was uridine diphosphoglucose. Since these pioneering studies, it has become increasingly apparent that, in addition to their role as precursors of nucleic acids, many acid-soluble nucleotides have roles in other metabolic processes. There are four groups of acid-soluble nucleotides related to the four principal ribonucleotides which occur in ribonucleic acid (as well as several, recently discovered, which contain deoxyribonucleotides). The adenosine nucleotides have been known for at least 25 years. Soon after the isolation of UDP-glucose, guanosine and cytidine nucleotides were also discovered as components of the acid-soluble fraction of tissues. Each of these types of nucleotide has a special metabolic role in addition to its role as a nucleic acid precursor. By way of introduction, the general types of compound for each nucleotide and their diverse metabolic functions will be described.

In the case of the adenosine nucleotides, there are, first of all, the oxidation-reduction intermediates, DPN, TPN and FAD.† Each of these compounds, as well as most of the others, can be considered as the acid anhydride of a mononucleotide, i.e. a substituted phosphoric acid, and another acid. For example, DPN is the anhydride of adenylic acid and nicotinamide ribose phosphate, and FAD is the anhydride of adenylic acid and riboflavin phosphate. These compounds are synthesized primarily by re-

* This paper and the one following it are edited transcriptions of two lectures given at Freiburg University in July, 1960, and as the McLaughlin Lectures at The University of Texas in October, 1960. Work in the author's laboratory has been generously supported by the U.S. Public Health Service (Grants NIAMD A-1158 and NIAID E-1902) and by the U.S. National Science Foundation (Grant G-7619), to whom I am greatly indebted. Department of Pharmacology, Washington University School of Medicine, St. Louis, Missouri.

† Documentation of the first half of this lecture would require a bibliography of several hundred references. Only a few references have, therefore, been provided, but the sources of the work described have been fully documented in several reviews (2–5), including one by the author (6).

action between a nucleoside triphosphate (ATP in the case of the adenosine nucleotides) and the acid which is to be activated (Equation 1). For example, a reaction

1. XTP + acid ⇄ XMP − acid + PP

between ATP and riboflavin phosphate leads to formation of the anhydride, flavin adenine dinucleotide (FAD), with the simultaneous splitting of inorganic pyrophosphate. This mechanism for synthesis of these acid anhydrides by means of displacement of inorganic pyrophosphate from a nucleoside triphosphate by another acid was first discovered by Kornberg during studies of DPN metabolism (2).

In addition to the oxidation-reduction intermediates, a number of other types of compounds are activated as derivatives of adenylic acid, e.g. amino acids. Adenyl-amino acids have in recent years been isolated and synthesized, and are believed to be intermediates in the biosynthesis of proteins. Another compound activated in this way is inorganic sulfate. Adenyl-sulfate is formed as the product of th eprimary reaction with ATP. In this latter case, however, the equilibrium is extremely unfavorable and in the living cell it is pulled by the phosphorylation of the adenylic acid moiety (Equation 2 and 3). The product is "active sulfate," 3′-phosphoadenosine 5′-phosphosulfate (PAPS). This compound has a role in biosynthesis of chondroitin sulfates and of other naturally occurring sulfate esters (Equation 4) (7).

2. ATP + sulfate ⇆ AMP − sulfate + PP
3. AMP − sulfate + ATP → PAPS + ADP
4. PAPS + acceptor ⇄ acceptor − S + PAP

The second group of compounds, to which we shall return in more detail, is the uridine nucleotides (3,6). These compounds are anhydrides of uridylic acid and a sugar phosphate, i.e. UDP-sugar compounds (Equation 5). A very large number of such compounds are now known, and they serve many important metabolic roles in transglycosylation reactions (Equation 6) and in transformations of one sugar to another.

5. UTP + glucose-1-phosphate ⇄ UDP-glucose + PP
6. UDP-glucose + fructose ⇄ sucrose + UDP

The third group of compounds is the guanosine nucleotides

(4,6). These nucleotides are also a means of activation of sugars. However, this group has not yet been very extensively studied. Only recently has the isoltaion of some new compounds stimulated interest in these compounds. The first known was GDP-mannose (8). Its precise metabolic role is still unknown, but it is presumably an intermediate in the synthesis of mannose-containing polysaccharides. GDP-*D*-mannose is also the precursor of GDP-*L*-fucose (9). Fucose (6-deoxy-*L*-galactose) is, therefore, synthesized by living organisms in the form of its nucleotide derivative. Fucose is a very important substance in the blood group substances, as well as in many other types of polysaccharides. GDP-*D*-mannose can also be transformed to GDP-colitose (10). Colitose is a 3,6-dideoxy-hexose (3,6-dideoxy-*L*-galactose), found in the O-endotoxin of several gram-negative bacteria. For example, it occurs in the O-endotoxin of some pathogenic *E. coli* strains, which are the causative agents of infantile diarrhea. The mechanism of synthesis of O-endotoxins and of their precursors are subjects now of very active investigation.

Finally, the compounds which are activated as derivatives of cytidylic acid, are, with one exception, alcohol (5,6). One alcohol which is activated as a cytidine nucleotide is choline (Equation 7). CDP-choline is a key intermediate in phospholipid synthesis (Equation 8) (5). Note that in the synthetic reaction a phosphoalcohol is transferred (Equation 8) and CMP is the

7. CTP + phosphocholine $\rightleftarrows$ CDP-choline + PP
8. CDP-choline + diglyceride $\rightleftarrows$ lecithin + CMP

second product, in contrast to synthetic reactions of uridine nucleotides where the sugar is transferred (Equation 6) and UDP is the second product. Similarly, ethanolamine is activated in this way for the synthesis of ethanolamine lipids. The synthesis of inositol lipids also takes place through a mechanism involving a cytidine nucleotide, although a somewhat more complicated mechanism is used than in the synthesis of choline and ethanolamine lipids (11). Two compounds which have so far been isolated only from bacteria are CDP-glycerol and CDP-ribitol (12). They are presumably intermediates in the biosynthesis of components of bacterial cell walls and of other bacterial heteropolymers. CMP-N-acetylneuraminic acid is a very unusual nucleotide in this group (13).

Similar derivatives of nucleotides found in deoxyribonucleic acid have been isolated recently although so far this group of compounds is very limited. Deoxy-CDP-choline and deoxy-CDP-ethanolamine were the first of these isolated (14,15). More recently, thymidine diphosphosugar compounds have been isolated (e.g. TDP-*L*-rhamnose, (16,17) TDP-*D*-fucose, (18,19) TDP-6-deoxy-*D*-glucose, (18,19) TDP-4-keto-6-deoxy-*D*-glucose, (19, 20) and TDP-mannose (21)). The occurrence of nucleotide derivatives which are so closely related chemically to deoxyribonucleic acid raises the question as to whether they are related functionally in any way to the genetic material.

A large number of small molecules are, therefore, activated for synthetic reactions as derivatives of a variety of nucleotides. Acids are activated as adenosine nucleotides, while sugars (aldehydes) are activated as guanosine, uridine or thymidine nucleotides and alcohols are activated as cytidine or deoxycytidine nucleotides.

In Figure 1 is shown the structure of one of these nucleotides,

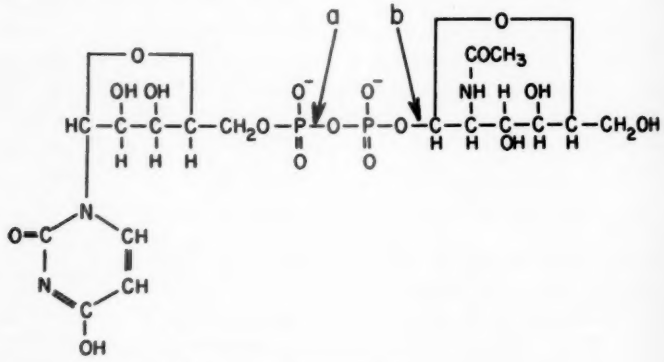

FIG. 1. Structure of UDP-acetylglucosamine.

uridine diphosphoacetylglucosamine (22). It is a precursor of acetylglucosamine-containing polysaccharides, such as hyaluronic acid. The acid anhydride bond at "a" is formed in the reaction between the nucleoside triphosphate and the sugar phosphate and the bond at "b" is split in the synthetic reaction involving the transfer of the sugar to a growing polysaccharide chain. The sugar can be considered as a fragment, activated by the nucleo-

tide for some synthetic process. Many sugars are activated in this way, and in Figure 2, the UDP-sugar compounds related to UDP-

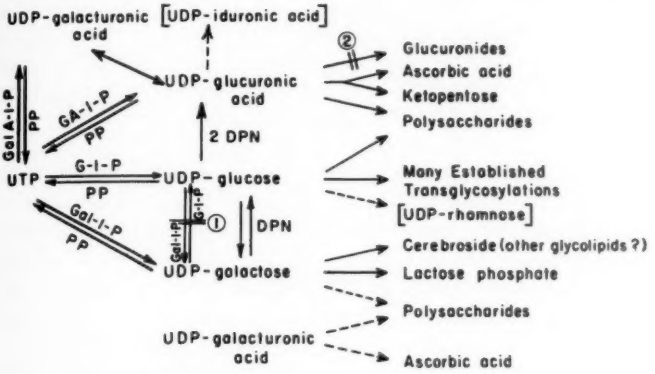

FIG. 2. Uridine diphosphohexoses.

glucose are shown. A primary reaction in this scheme is the reaction of glucose-1-phosphate with uridine triphosphate to give UDP-glucose with formation of inorganic pyrophosphate (Equation 5). Once activated in the form of its nucleotide derivative, the glucose moiety can be modified in a number of ways. UDP-glucuronic acid, UDP-galacturonic acid, and UDP-galactose are formed through transformation of UDP-glucose, and each of these sugar-containing nucleotides can participate in a synthetic reaction. For example, UDP-glucuronic acid is used in the synthesis of glucuronides. UDP-glucose is an intermediate in the biosynthesis of sucrose in plants (Equation 6) and in recent years it has been shown that glycogen is not synthesized directly from glucose-1-phosphate but rather from UDP-glucose. UDP-galactose is used in the synthesis of lactose and of cerebrosides in brain. UDP-galacturonic acid undoubtedly has many important metabolic roles which have not yet been adequately described. Two congenital defects in uridine nucleotide metabolism occur in man. One of these is a defect in formation of UDP-galactose from galactose-1-phosphate (galactosemia) and the other one is a defect in the transfer of glucuronic acid from UDP-glucuronic acid to bilirubin (congenital non-hemolytic bilirubinemia).

Glucuronide synthesis provides an excellent example of a

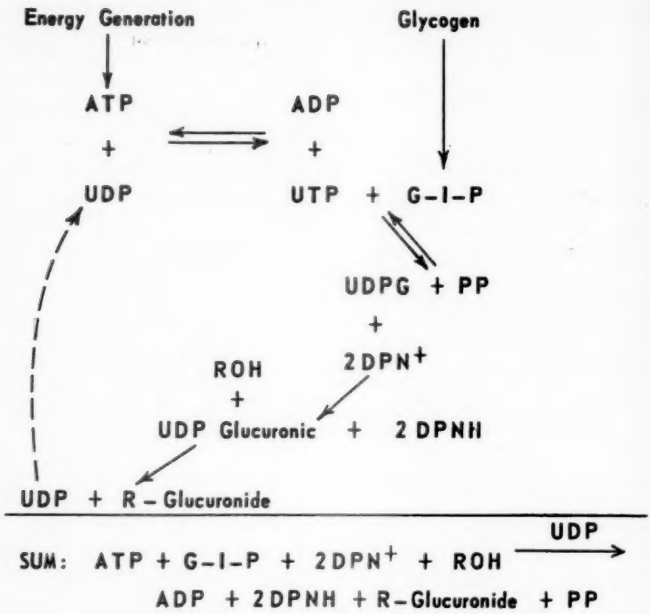

FIG. 3. The cyclic mechanism of glucuronide synthesis.

typical reaction cycle for one of these nucleotides (Figure 3). This is a mechanism by which many drugs and other foreign organic compounds as well as normal metabolites are conjugated prior to excretion. First, uridine triphosphate is formed from uridine diphosphate. Next, glucose-1-phosphate is activated by reaction with UTP. Then the sugar moiety of UDP-glucose is modified, in this case by oxidation, to UDP-glucuronic acid. Finally, the modified sugar is transferred to an acceptor (e.g. bilirubin) with formation of a glucuronide. The UDP which is formed as the second product of this reaction can then cycle again. The essential features of this cycle, to which we will return in the second lecture, are 1. phosphorylation, 2. activation of the sugar, 3. modification of the sugar, and finally, 4. transfer of the sugar to an acceptor in the synthetic reaction.

To comment briefly on the reaction by which glucuronic acid is formed from UDP-glucose (Equation 9), (23) it is one of two

examples of an oxidation which involves a four-electron transfer,

9. UDP-glucose + 2 DPN$^+$ → UDP-glucuronic acid + 2 DPNH + 2 H$^+$

and for that reason may be mechanistically very interesting. The enzyme which catalyzes the oxidation of the amino alcohol, histidinol, to the amino acid histidine (24), is the only other known example of such an enzyme. All other known biological oxidations involve either one- or two-electron transfers.

In addition to serving as a donor of glucuronic acid for the synthesis of glucuronides, UDP-glucuronic acid has another important metabolic role as a precursor of free glucuronic acid, both for ascorbic acid synthesis and for a third cycle of glucose catabolism which occurs in man as well as in other animals (Figure 4).

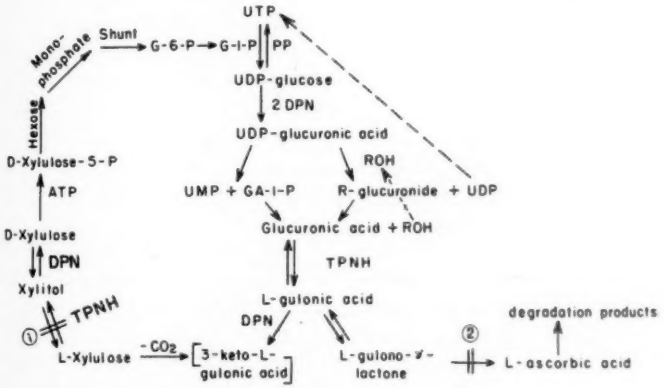

FIG. 4. The uridine nucleotide shunt.

This cycle is known both as the uridine nucleotide shunt and as the glucuronic acid pathway. Again, there are two defects in enzymes of this cycle which occur in human beings. One of these defects is the inability of the species to form ascorbic acid, which man shares with monkeys and guinea pigs. The other defect is in the conversion of *L*-xylulose to xylitol, a benign condition known as congenital pentosuria. The consequence of this defect is the excretion of very large amounts of *L*-xylulose. The *L*-xylulose originates from free glucuronic acid formed from the uridine nucleotide.

Plants also have mechanisms for activating sugars as nucleotide derivatives. One of these mechanisms in plants is the activation of the pentoses, xylose and arabinose, as UDP-derivatives for the synthesis of xylans and arabans, important constituents of many plants (Figure 5) (25).

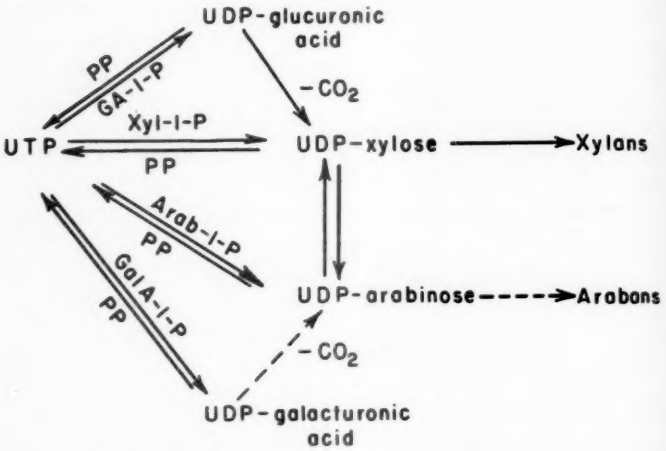

FIG. 5. Uridine diphosphopentoses.

Finally, the acetylamino sugars comprise a third group of sugars which are activated as uridine nucleotides (Figure 6).

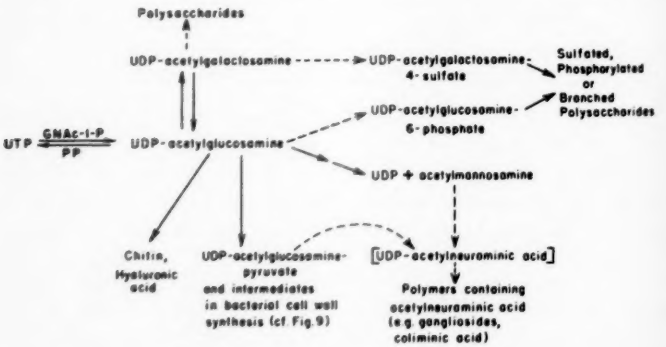

FIG. 6. Uridine diphosphoacetylamino sugars.

An important reaction is that between UTP and acetylglucosamine-1-phosphate, leading to formation of UDP-acetylglucosamine, which can then be modified in a number of ways. First of all, it can be transformed to UDP-acetylgalactosamine. Further reactions, which are not yet identified, lead to synthesis of UDP-acetylgalactosamine-4-sulfate and of UDP-acetylglucosamine-6-phosphate,* two unusual compounds which have been isolated from hen oviduct. These compounds, as well as UDP-acetylglucosamine, are presumably precursors of various polysaccharides, including the chondroitin sulfates and hyaluronic acid. At the bottom of Figure 6 is shown a group of compounds, which are precursors of bacterial cell walls and which form the subject of the second lecture.

With this background, we can discuss current knowledge of the mechanism of synthesis of the chondroitin sulfates and, thereby, illustrate the role of uridine nucleotides and of other nucleotides in the biosynthesis of polysaccharides. Some interesting data relating to polysaccharide structure also grew out of these studies of the biosynthesis of chondroitin sulfates.

These studies began with the isolation of UDP-acetylgalactosamine-4-sulfate from the oviduct of laying hens (26). The acetylgalcatosamine-4-sulfate moiety, activated in the form of the uridine nucleotide, is presumably a precursor of some sulfated polysaccharide. To consider a possible function of this compound, it is necessary to consider the function of the oviduct, a very complicated assembly line. The yolk enters from the abdominal cavity through the infundibulum and passes first into the region where the albumin is secreted, a region about 20 cm. in length, by far the largest region in the oviduct. Then the egg passes into a very narrow area, the isthmus, about two centimeters long in a hen, in which the inner egg shell membranes are secreted around the albumin. These membranes are secreted in a period of about an hour. Their structures are still not known, but they do contain a sulfated mucopolysaccharide of some kind. Calcification takes place in the third region of oviduct (uterus). The terminal region (vagina) is the region where the smooth outer surface of the egg

* It has been found recently that UDP-acetylglucosamine-6-phosphate (26) is a degradation product of the nucleotide actually present in hen oviduct. The structure of this compound, UDP-acetylglucosamine-6-phospho-1-*D*-galactose was deduced independently in two laboratories (27,28). The galactose moiety in the nucleotide is extremely labile.

(cuticle) is secreted, immediately before the hen lays the egg. In order to find out where sulfate metabolism occurred in this complicated factory, S^{35}-inorganic sulfate was given to living hens. It was taken up only in two discrete regions of the oviduct, the isthmus and the vagina, in each of which shell membranes are synthesized (Figure 7). In both of these regions, S^{35} was

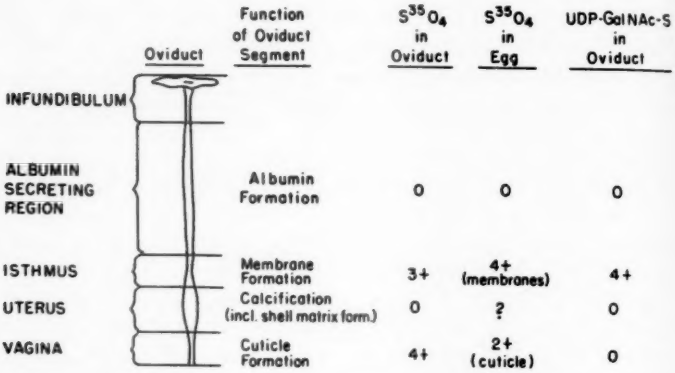

FIG. 7. Sulfate metabolism in the oviduct of the laying hen (29).

located in the glandular epithelium. Eggs laid by these hens were also examined; both the inner shell membranes and the cuticle were radioactive (29).

The distribution of the sulfated uridine nucleotide in the segments of oviduct was examined next. This nucleotide was found only in the isthmus. Its concentration in this region is about 5 micromoles/gram, an extremely high concentration for a nucleotide in a tissue. When a paper chromatogram was made from a hot water extract of isthmus, the sulfated uridine nucleotide was the only ultraviolet-absorbing compound which was detected. These data suggested that isthmus was an excellent tissue with which to study the synthesis of sulfated mucopolysaccharides.

At about this time Robbins and Lipmann (30) had isolated phosphoadenosine phosphosulfate. It seemed plausible that PAPS might be the sulfate donor for the synthesis of UDP-acetylgalactosamine-sulfate and that the reaction might be a direct sulfation of UDP-acetylgalactosamine. S^{35}-labeled phosphoadenosine phosphosulfate was prepared usnig enzymes isolated from isthmus.

Incorporation of S^{35} from $PAPS^{35}$ into chondroitin sulfate was studied in the belief that the reaction sequence would lead through the sulfated uridine nucleotide. When $PAPS^{35}$ was incubated with enzyme from isthmus, the major reaction was hydrolysis of PAPS to inorganic sulfate. If, however, an acceptor, such as chondroitin sulfate A, was also added, a new sulfated product was formed (Table 1) which remained at the origin on

TABLE 1

Synthesis of Sulfated Mucopolysaccharides from $PAPS^{35}$ (31)

Exp. 1: System with ChS-A as acceptor	c.p.m. in polysaccharide	Exp. 2: Various acceptors	c.p.m. in polysaccharide
1. Complete	3320	ChS-A	1750
2. Minus ChS-A (added after incubation)	30	ChS-B	1330
3. Minus $PAPS^{35}$ (added after incubation)	25	ChS-C	1060
4. Boiled enzyme control	30	Heparitin sulfate	1000
5. APS^{35} substituted for $PAPS^{35}$	100	Heparin, hyaluronic acid, glycogen or charonin sulfate	40 to 50

In these experiments S^{35} sulfate was transferred from $PAPS^{35}$ of high specific activity to various acceptors, catalyzed by an enzyme preparation from the isthmus of hen oviduct. (31) Data are recorded as c.p.m. incorporated into polysaccharide acceptor under the conditions employed. In experiment 1, omission and substitution of various components of the system was studied. In experiment 2, the relative rates of sulfation of various acceptors was examined.

a paper chromatogram (Figure 8) and which was then recovered and shown to be radioactive chondroitin sulfate A (31). A number of polysaccharides would serve as acceptors in this system, viz: chondroitin, chondroitin sulfate A, chondroitin sulfate B, chondroitin sulfate C, heparitin sulfate, and, finally, a chondroitin sulfate which had been isolated in Japan from shark cartilage and which was believed to be identical to chondroitin sulfate C (Figure 9).* However, the apparent K_m and V_{max} for this latter compound were different from those for chondroitin sulfate C. These data suggested that the compound might not be identical with chondroitin sulfate C. Other data (see below) have proven that it is, in fact, a novel form of chondroitin sulfate.

In considering the mechanism by which sulfate was incorporated into the polysaccharides in this system, the idea that the

* It is now known, however, that several different sulfotransferases in the preparations are responsible for the sulfation of different polysaccharides (32).

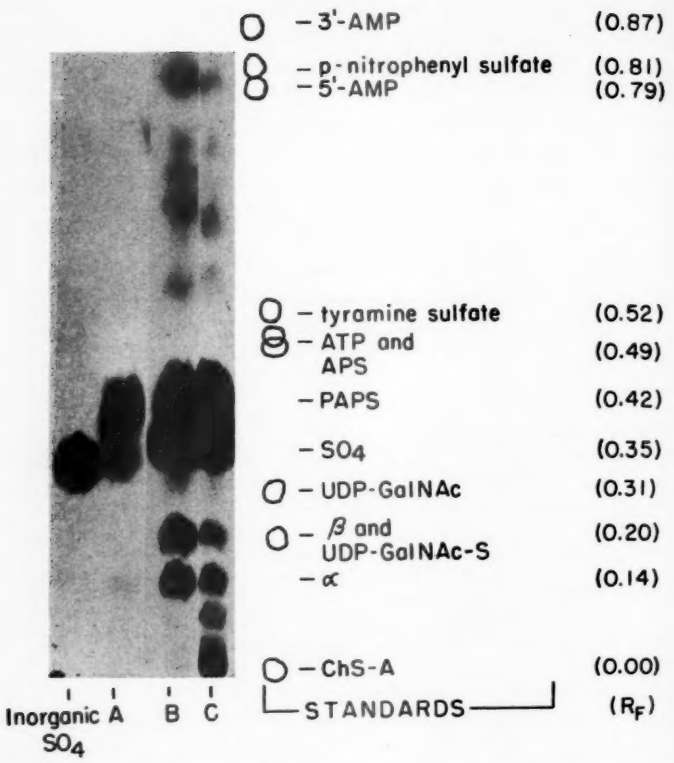

FIG. 8. Radioautogram of a chromatogram of incubation mixtures containing $PAPS^{35}$ and an enzyme preparation from isthmus of hen oviduct (31). A Enzyme boiled before incubation B. Incubated in the absence of added acceptor C. Incubated with 50 μg of a chondroitin sulfate A preparation, partially digested with hyaluronidase. The radioactive compound at the origin is the polymer; those between R_f 0.0 and 0.14 are sulfated oligosaccharides. If undigested polymer was used, the only new product observed in C was that at the origin.

sulfated uridine nucleotide (formed from an endogenous acceptor) was an intermediate in the incorporation and that the polysaacharide served as an acceptor for acetylgalactosamine-sulfate (transferred from the uridine nucleotide) was attractive (equation 10). However, a second possibility had to be considered, viz: that the uridine nucleotide was not an intermediate

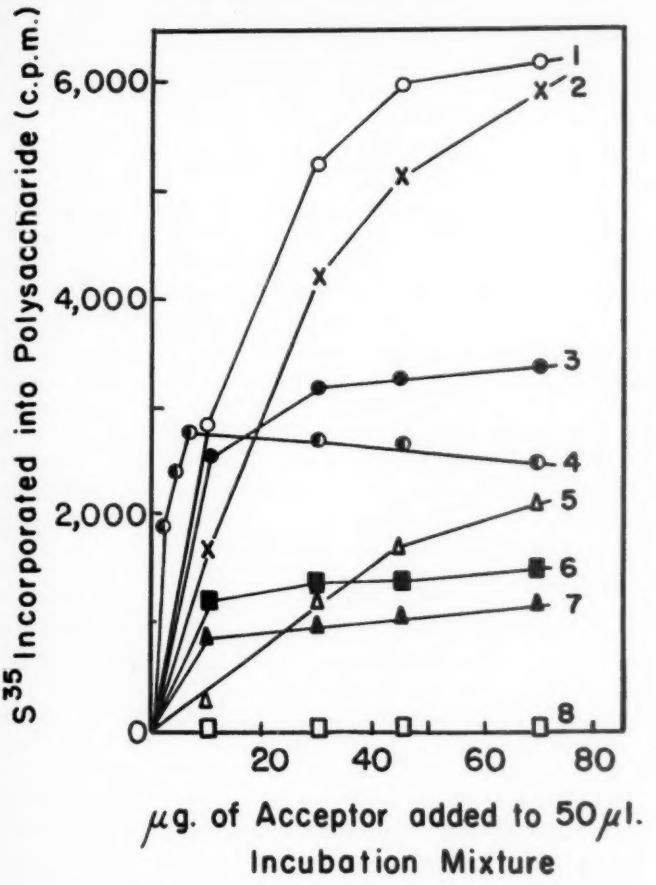

FIG. 9. Relationship between concentration of various acceptors and reaction velocity (31). The acceptors employed were: 1, chondroitin sulfate C; 2, chondroitin from bovine cornea; 3, chondroitin sulfate A; 4, chondroitin sulfate B; 5, chondroitin prepared by chemical desulfation of chondroitin sulfate A; 6, heparitin sulfate; 7, chondroitin sulfate from shark cartilage; 8, glycogen, heparin, keratosulfate, hyaluronic acid, or charonin sulfate.

but that the polysaccharide itself was the primary acceptor in the transsulfation (equation 11).

10. Polysaccharide $\xrightarrow[\text{PAPS} \uparrow \text{UDP-GalNAc}]{\text{UDP-GalNAc-S}}$ Polysaccharide-GalNAc-S

11. Polysaccharide $\xrightarrow{\text{PAPS}}$ Polysaccharide-S

It was impossible to study this problem using the large polymers as acceptors because the simple addition of S^{35}-sulfate to these high molecular weight substances could not be distinguished from addition of acetylgalactosamine-S^{35}-sulfate. The sulfation of low molecular weight oligosaccharides derived from chondroitin and from chondroitin sulfate A by enzymatic hydrolysis was, therefore, investigated. In this case it was relatively easy to distinguish the possible products of sulfation by paper chromatography. For example, if the tetrasaccharide containing no sulfate residues was used as an acceptor, the possible products, tetrasaccharide monosulfate or pentasaccharide monosulfate, could be easily distinguished. To prepare the sulfated products, enzyme and $PAPS^{35}$ were incubated with various oligosaccharide acceptors. The incubation mixtures were then chromatographed, and in each case a new radioactive product was formed which had a slower mobility than the acceptor itself. This radioactive product was eluted from the chromatogram and its mobility determined on paper chromatography and on paper electrophoresis together with authentic standards. In each case, it was found that the sulfation reaction was a simple transfer of sulfate from the donor to oligosaccharide. Acetylgalactosamine and acetylgalactosamine monosulfate were also both sulfated, with the formation of acetylgalactosamine monosulfate or acetylgalactosamine disulfate, respectively, as the products (Figure 10).

The relative rates of sulfation of all of the oligosaccharides which were isolated is shown in Figure 11. Although most of these compounds were sulfated at rates which were relatively slow, the trisaccharide (GalNAc-GA-GalNAc) and the pentasaccharide (GalNAc-GA-GalNAc-GA-GalNAc) were sulfated at a rate comparable to that of the polymer itself. This may be an important point. The even-numbered oligosaccharides all contained glucuronic acid on the non-reducing end of the oligosaccharide chain. The odd-numbered oligosaccharides were pre-

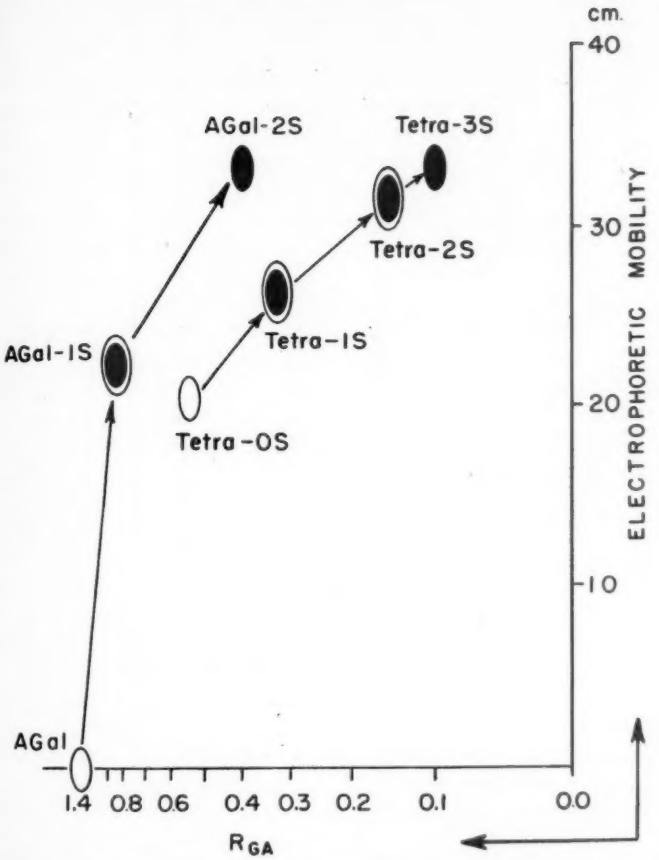

FIG. 10. Sulfation of monosaccharides and tetrasaccharides (31). The position on paper chromatography in butanol acetic acid-water (R glucuronic acid) and on paper electrophoresis is shown. The position of the added acceptor is indicated by an open symbol and that of the radioactive product by an arrow pointing to a black symbol.

pared from the even-numbered compounds by removal of the terminal glucuronic acid residue with a β-glucuronidase, yielding oligosaccharides with an acetylgalactosamine residue on the non-reducing end. These odd-numbered oligosaccharides were extraordinarily efficient as acceptors in the sulfation system. It

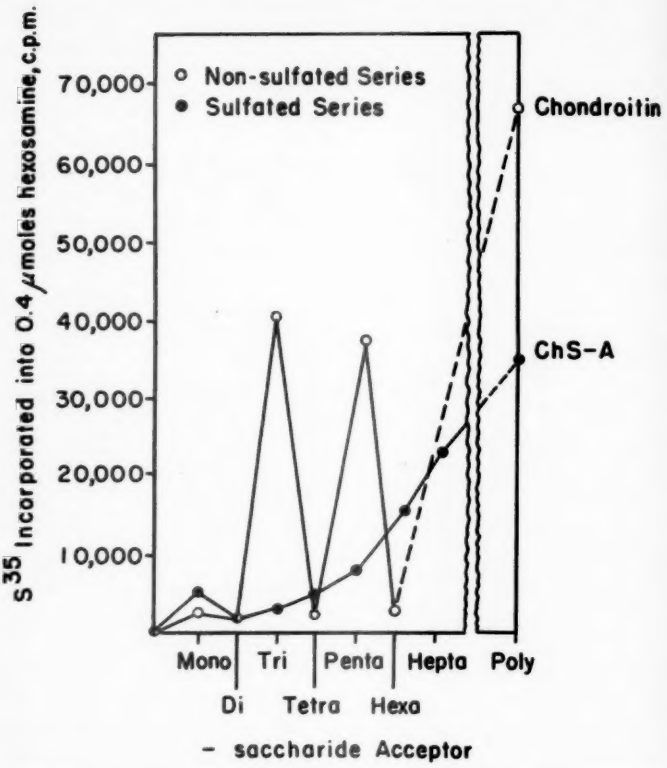

FIG. 11. Relationship between chain length of the acceptor and the velocity of sulfate transfer (31).

is possible, therefore, that at least one mechanism for the introduction of sugars and of sulfate into polysaccharide is sulfation of an acetylgalactosamine residue on the non-reducing end of a growing polysaccharide chain. However, the role of the sulfated uridine nucleotide still remains to be elucidated, and there may be several mechanisms for the introduction of sugars and of sulfate into polysaccharide chains.

Two interesting aspects of the structure of chondroitin sulfates grew out of these enzymatic studies. As you remember, acetylgalactosamine itself was a sulfate acceptor. The product was an

acetylgalactosamine monosulfate which was electrophoretically identical to acetylgalactosamine monosulfate derived from the uridine nucleotide by hydrolysis as well as to acetylgalactosamine sulfate prepared by chemical sulfation of acetylgalactosamine with chlorosulfonic acid. However, on paper chromatography of these three acetylgalactosamine monsulfates, the compound from the uridine nucleotide had a faster mobility than the compound which had been prepared chemically. The enzymatically synthesized product was identical to that derived from the uridine nucleotide.

The structure of these acetylgalactosamine monosulfates was examined (33). Periodate oxidation with measurement of both periodate consumption and formaldehyde liberation will distinguish between all of the possible isomeric acetylgalactosamine sulfates. It was established that the acetylgalactosamine monosulfate derived from the nucleotide was acetylgalactosamine-4-sulfate while the acetylgalactosamine monosulfate which was prepared by chemical sulfation was acetylgalactosamine-6-sulfate. The acetylgalactosamine monosulfates were then isolated from several polysaccharides, and by comparison with the now characterized materials its was established that the compound from chondroitin sulfate A or B was the 4-sulfate while that from C was the 6-sulfate, thus confirming by direct chemical isolation what other invsetigators had previously concluded from other kinds of studies (Table 2).

The second serendipic aspect of this investigation of the mechanism of chondroitin sulfate synthesis was the discovery of a new form of chondroitin sulfate, which was called chondroitin sulfate D, a compound first isolated by Egami and co-workers in Japan from shark cartilage (34). As already mentioned, this compound was not identical to chondroitin sulfate C as an acceptor for the sulfotransferase, and the compound had furthermore been found to contain about 22% sulfate (*i.e.*, 1.3 sulfate residues per acetylgalactosamine residue). To investigate its structure, an enzyme from *Proteus vulgaris* (35) was employed by Suzuki (36). This enzyme catalyzes an essentially quantitative hydrolysis of chondroitin sulfates to disaccharides. The reaction is an elimination reaction, rather than a hydrolysis, so that the product is a $\triangle$ 4,5-unsaturated disaccharide (36). When the products of hydrolysis of various chondroitin sulfate preparations were separated by

TABLE 2

Identification of Acetylgalactosamine Monosulfates (33)

	R_f in Isobutyric acid: NH_4OH	Moles of HCHO formed/mole*	$E_{540}m\mu$ in Morgan-Elson reac.	Infrared peaks**
1. GalNAc-4-S from uridine nucleotide	0.52	0.92	200	695, 860, 890, 920
2. GalNAc-6-S, chemically synthesized	0.44	0.05	10,000	775, 820, 992
3. GalNAc-S from ChS-A	0.53 (trace at 0.44)	0.62†	1,800†	695, 860, 890, 920
4. GalNAc-S from ChS-B	0.52	0.75†	1,120†	695, 860, 890, 920
5. GalNAc-S from ChS-D	0.44	0.03	10,000	780, 815, 992

* After periodate oxidation at 25° for 20 hours. GalNAc-4-S consumed 1 mole of periodate while GalNAc-6-S consumed 4 moles.

** Peaks in the infrared spectra between 660 and 1000 cm^{-1}.

† These values suggest that these samples, which are predominantly GalNAc-4-S may contain 10–15% of GalNAc-6-S.

paper chromatography, chondroitin sulfate D yielded an unexpected product which was not formed on hydrolysis of either chondroitin sulfate A or of chondroitin sulfate C .A small amount of a similar product was formed also on hydrolysis of a preparation of chondroitin sulfate B. However, these two compounds could be separated by paper chromatography, and are believed to be isomers. On analysis each of these compounds contained two sulfate residues per disaccharide unit, which was reflected in a very rapid electrophoretic mobility of the disaccharide (Figure 12). Various data suggested that one sulfate residue of both of these compounds is located on the 2- or 3-position of the uronic acid moiety. For example, the periodate consumptions of the disulfated disaccharides are very much reduced, indicating that the glucuronic acid moieties are substituted in some manner, presumably by sulfate (Figure 13). The other sulfate residue is on the acetylgalactosamine. The compound from chondroitin sulfate D has a sulfate on the 6-josition of acetylgalactosamine while that from chondroitin sulfate B may be on the 4-position of the acetylgalactosamine moiety.

The mechanism by which uridine nucleotides and other nucleotides participate in the biosynthesis of sulfated mucopolysaccharides is, therefore, a vrey complicated problem. These compounds can contain at least four types of sulfate residues

FIG. 12. Relative electrophoretic mobilities of unsaturated non-sulfated disaccharide (ΔDi-OS), unsaturated monosulfated disaccharide (ΔDi-6S) and unsaturated disulfated disaccharide (ΔDi-DiS_D) derived from shark cartilage chondroitin sulfate and of the unsaturated disulfated disaccharide (ΔDi-diS_B) derived from a chondroitin sulfate B preparation (36).

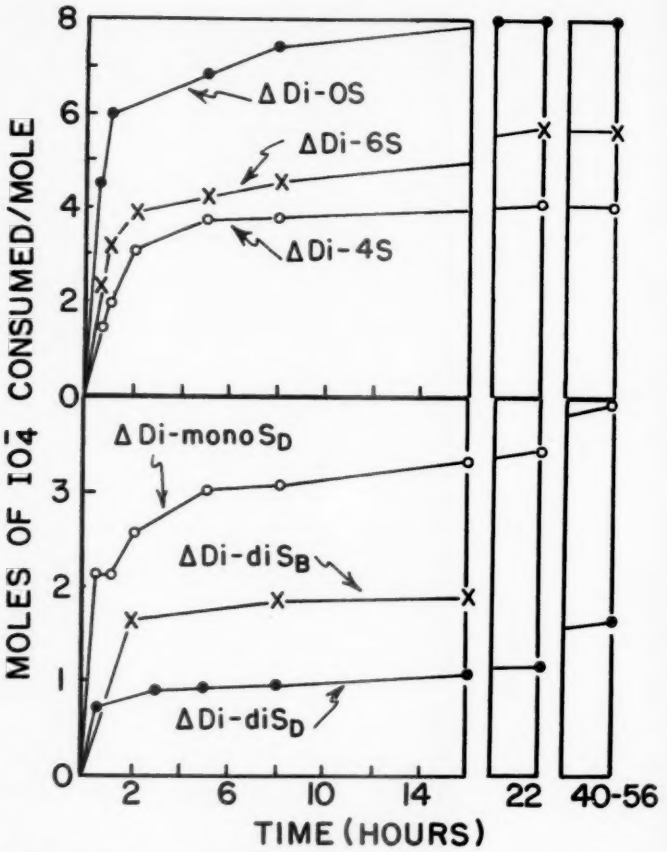

FIG. 13. Rate of periodate consumption of unsaturated disaccharides (36). The low periodate uptake of the disulated disaccharides (ΔDi-diS$_D$ and Δ Di-diS$_B$) relative to the monosulfated disaccharides (ΔDi-monoS$_D$, ΔDi-4S and Δ Di-6S) and non-sulfated disaccharide (ΔDi-0S) should be noted. Note the difference in scale of the ordinate for the two sections of the figure.

(4-0-, 6-0- or N-sulfate on the amino sugar and sulfate on the uronic acid) as well as several different sugars. Several types of mechanisms undoubtedly exist both for introduction of sulfate and for introduction of sugars into the polysaccharide chains. Undoubtedly there are more surprises to come, both in terms of

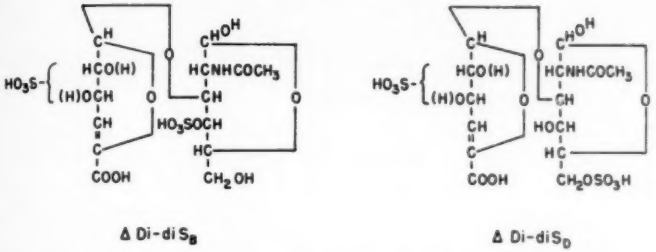

FIG. 14. Proposed structures of unsaturated disulfated disccharides from shark cartilage chondroitin sulfate (ΔDi-diS$_D$) and from a chondroitin sulfate B preparation (ΔDi-diS$_B$) (36).

biosynthetic mechanisms and in terms of structure. With the discovery of a number of nucleotide interemediates, it is hoped that more rapid progress can be made in study of the biosynthesis of the chondroitin sulfates as well as of other biologically important heteropolymeric substances.

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STRAIN DIFFERENCES AND THE RELATION OF AGING TO SALT SUSCEPTIBILITY IN MICE[a]

RAYMOND F. BLOUNT AND ISABEL H. BLOUNT[b]

Far-reaching morphological and physiological effects are brought about by the administration of high levels of NaCl, and length of survival is affected. These effects are less pronounced when the NaCl is administered with food than when given in water. In the work of Meneely and associates (10, 11) high salt was administered to rats as part of the diet. Heller (6) gave rats salt in drinking water but in general at low percentages, while Sapirstein, Brandt and Drury (13) used two per cent. Little other work has been done with higher salt administered in this manner.

There are differences in the response to NaCl toxicity by various animals. Dipodomys and Meriones, desert rodents, excrete a very concentrated urine and conserve water, as shown by Howell and Gersh (8), Schmidt-Nielsen, Schmidt-Nielsen and Schneiderman (15) and by Howe and Jewell (7). Schmidt-Neilsen (14) also showed that Dipodomys, the kangaroo rat, is able to drink sea-water. These animals also withstand high salt in the diet somewhat better than does the ordinary laboratory rat, as shown by Nichols (12). As was briefly reported earlier (3, 4) we have had available two strains of mice with contrasting susceptibility to NaCl toxicity. The present study deals with the effect of high NaCl administered in drinking water on the survival time of these two strains at various ages.

Materials and Methods

Mice of two strains were used in the work. One, referred to as the resistant strain is designated IHB, and is a locally developed inbred strain now in more than the twenty-fifth generation of brother-sister matings. The original stock was from the "All Purpose Strain" of mouse from Rockland Farms. The so-called susceptible strain is a substrain of the NH mouse also maintained in our laboratory by brother-sister matings. In all about 1,700 mice have been

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entered in this report. Several hundred additional mice, with the same treatment and showing the same results, have been used for other purposes.

The mice were numbered with ear marks and placed on 4% NaCl as drinking water. Control animals were placed on distilled water. The food was one of the ordinary pellet forms. The animal room had controlled temperature, although a fluctuation of five degrees was sometimes noted. The date of death was recorded for each animal. Test animals and their controls were weighed at intervals and their general appearance and behavior noted.

Results

The general effect of the experiment was a reduction in weight, with debility, and as the terminal stage was approached there was often muscular tremor. A test animal of the resistant strain after a year and 44 days on 4% NaCl is shown in Figure 1 (B) together with its control (A). It weighed 11 grams and its control 35 grams. In contrast an individual of the susceptible strain shown in Figure 2 (B) had been on salt only 11 days and

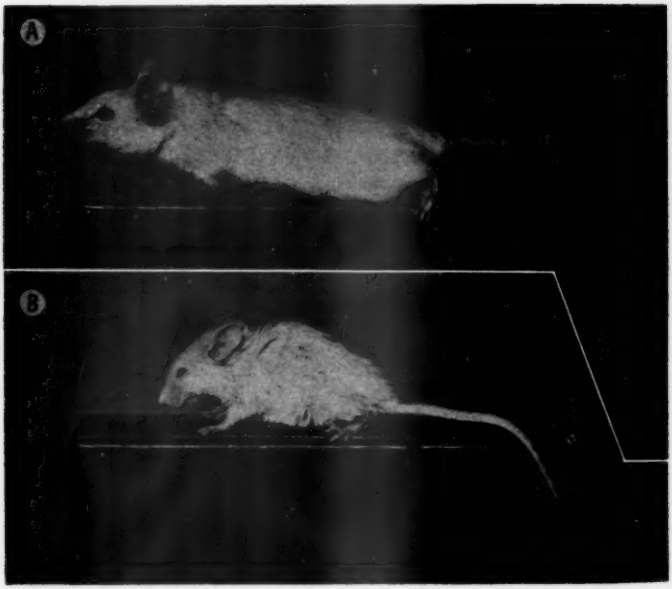

FIG. 1. A set of animals of the resistant strain (IHB). One year and 44 days after the beginning of the experiment. A.—Control animal, weight 35 grams. B.—Test animal on 4% NaCl in drinking water, weight 11 grams.

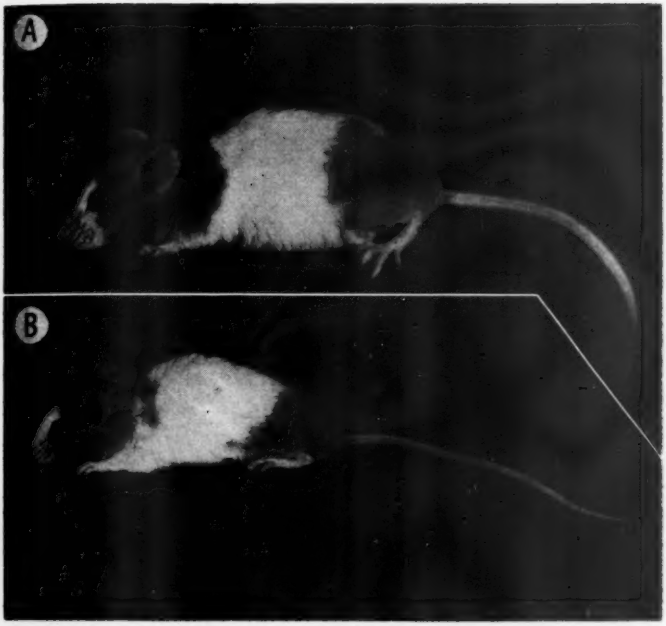

FIG. 2. A set of animals of the susceptible strain (NH). Twelve days after the beginning of the experiment. A.—Control animal, weight 22 grams. B.—Test animal, weight 12 grams. Photograph taken one day previous to death.

weighed 12 grams whereas the control mouse (A) weighed 22 grams. The test animal died on the twelfth day.

Weight curves made for typical test animals of both strains, together with their controls, are shown in Figure 3. For each strain two different starting ages are shown. Most resistant to the NaCl was the IHB animal (curve A) started at 53 days of age and 19 grams in weight. This mouse survived for one year and 34 days, it weighed 13 grams at the time of death. The control (B) by that time had not only attained its adult weight but showed a continued rise through the accumulation of fat. An NH animal (E), started at the same age as the IHB animal and weighing 16 grams, died after only 45 days, at which time its weight was 11 grams. Its control then weighed 24 grams. The older IHB test animal (C) did better than the young NH. Starting on NaCl at 360 days of age and at 35 grams in weight, it de-

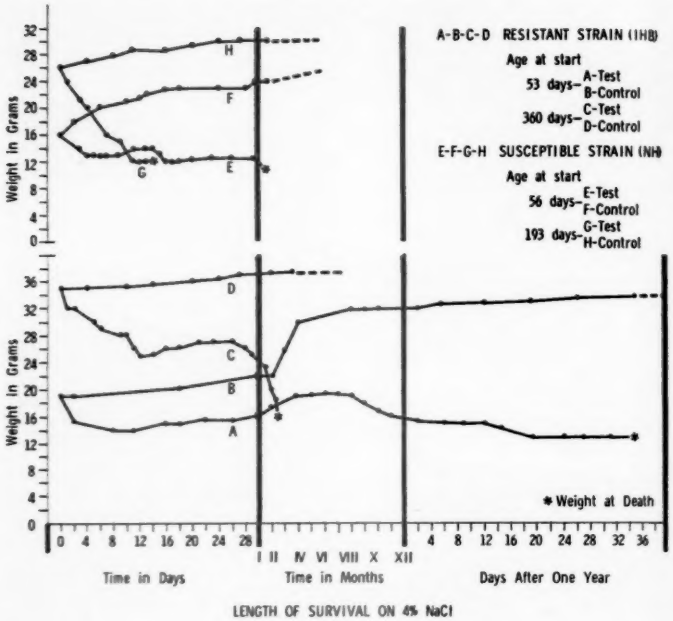

FIG. 3. Weight curves for typical animals. Compare A with E for difference of strain susceptibility. Compare C with A, and G with E for age differences.

clined rather rapidly but survived between 3 and 4 months and weighed 15 grams at death. The growth of the control (4) had levelled off at 37 grams. Shortest-lived of all was the older NH test animal (G) which was put on salt at 193 days of age with a weight of 26 grams. It died on the fourteenth day weighing 11 grams, when its control weighed 28 grams.

For each test animal the weight curve showed a considerable decline at first, while the animal was becoming dehydrated. In all but the older NH (G) a varying amount of adjustment took place, so that the decline became more gradual or there were periods of increased weight, before the final decline. During the latter, animals often shrank to 11 grams or less.

The difference in susceptibility of the two strains of mice is readily evident if the effect upon animals of approximately the same age (Table 1) is compared. One hundred sixty mice of the

TABLE 1

Comparison of the Survival of the Two Strains of Mice

Placed on 4% NaCl between 22 and 40 days of age

Number of Animals — Resistant Strain (IHB)—160; Susceptible Strain (NH)— 50

Days on Salt	Survival in %		Days on Salt	Survival in %	
	IHB	NH		IHB	NH
5	99	80	210	61	0
10	95	38	240	53	0
15	93	34	270	46	0
20	93	18	300	35	0
25	93	14	330	28	0
30	91	12	360	18	0
40	89	8	390	9	0
50	89	6	420	7	0
60	87	6	450	1	0
90	84	0	480	1	0
120	75	0	510	1	0
150	70	0	540	0	0
180	66	0	570	0	0

resistant strain (IHB) were placed on 4% NaCl in drinking water when between 22 and 34 days of age, and 99% were alive after 10 days and 91% after 30 days. Sixty-one per cent of them were still alive after 210 days and 7% lived 420 days. In contrast, of 50 animals of the susceptible strain (NH) placed on salt between the ages of 27 and 40 days, only 38% survived for 10 days, 12% for 30 and 6% for 60 days. None lived for 90 days.

The length of time that animals could survive decreased inversely with the advance in the age at which the salt treatment was started. Thus of 90 animals of the resistant strain put on salt (Table 2) when relatively young (20–30 days), 44 were still living 120 days later and 17 after a year. One lived for 510 days. Of the 103 mice started on salt when they were over a year old (361–450 days) only three were living 60 days afterwards. Between these two groups the results showed a remarkably uniform curve of survival. The older the animals were when the experiment started, the shorter was the average survival.

The susceptible strain showed a similar pattern of survival, but the deleterious effect of the salt appeared at younger age

TABLE 2

Age and Susceptibility of the Resistant Strain

Days Age at Start	21–30	31–60	61–90	91–180	181–270	271–360	361–450	451–540	541–630	631–668
Number Started (1154)	90	345	81	156	147	183	103	45	2	2
Days after Start	Number Surviving									
10	59	341	67	126	82	102	36	16	0	0
20	52	321	50	54	25	24	5	1		
30	51	296	41	37	17	11	3	1		
60	49	281	38	28	12	2	3	0		
90	47	253	35	16	10	1	0			
120	44	240	34	14	8	0				
150	39	205	34	12	6					
180	38	176	30	5	6					
210	37	156	24	2	5					
240	31	138	23	2	2					
270	29	121	18	1	1					
300	29	85	10	1	0					
330	26	58	7	1						
360	17	37	5	0						
390	11	16	4							
420	8	7	4					Control		
450	2	2	0					Median Survival		
480	1	1						Time on H_2O		
510	1	0						547 Days		
540	0									
Median Survival Time	240	225	195	135	105	25	20	15		

levels (Table 3). Thus of 43 animals put on salt when they were between 27 and 40 days old, only one survived for 70 days. Of the 43 started when 121 to 274 days of age (last 3 columns) none survived as long as 40 days.

There is some evidence that animals less than 30 days old are less resistant than those at 30 days of age. A comparison of the figures in the first column of Table 2 with those in the second column shows a smaller proportion of animals surviving for 10 days than might be expected, although the group as a whole lived very well. It appears that if an animal of this age group can survive for 30 days, it has greater tolerance than one started at

TABLE 3

Age and Susceptibility of the Susceptible Strain

Dage Age at Start	27–40	41–50	51–60	61–70	71–80	81–90	91–120	121–150	151–210	211–270
Number Started (299)	43	45	34	37	35	31	31	20	15	8
Days after Start	Number Surviving									
10	17	26	22	25	24	19	21	11	6	3
20	9	15	10	11	10	1	1	1	0	0
30	6	11	9	6	7	1	1	1		
40	4	4	6	0	4	1	0	0		
50	3	0	1	.	1	0				
60	3	.	0	.	1					
70	1	.	.	.	0					
80	0									
Median Survival Time	35	20	25	15	30	20	15	15	6	3

Control—Median Survival Time on distilled H_2O—118 Days

an older age. The same thing is true of the susceptible strain (Table 3).

Discussion

The effect of salt upon animals and upon their survival is dependent upon the method of administration as well as the concentration. The intake can be greater if given as a part of the diet and water is freely available. This has been demonstrated by the work of Meneely *et al.* (10,11) in which there was polydipsia and polyuria. Although the majority of the rats remained in excellent health with the percentage of NaCl in the diet as high as 8.9, growth was decreased and the length of life was reduced in proportion to the various salt levels. There was spontaneous edema in sporadic cases (18%), followed by a rapid decrease in weight, and death (11). It should be noted that we have never found edema in mice with 4% salt in drinking water. Tucker *et al.* (16) administered as high as 21 per cent NaCl in the food. This led to death in about six to eight months as compared to two and a half years in the control animals.

When NaCl was given to rats in the drinking water Heller (6)

noted deleterious effects on reproduction, and death when the level was above 1.5 or 1.7 per cent, which he considered to be the maximum that could be used with safety. In acute experiments on hypertension Friedman *et al.* (5) used sodium chloride by gavage as a tolerance test, and they pointed out the necessity of keeping the salt load within the capacity of the rat to handle it. Sapirstein, Brandt and Drury (13) also gave rats salt in drinking water, but at only a two per cent concentration and for a period of only six weeks. They noted the relaitvely greater effect when administered in water, in that there was an increase in heart weight and that hypertension was produced. These effects had not been brought about by an even higher intake of salt in food. We have found an increase in the heart weight in our mice on salt (3). As yet blood pressures have not been taken. Binet *et al.* (2) gave 1.6% NaCl in drinking water to rats conditioned by being raised on a high salt intake in food. They noted that these animals were more resistant to high salt than the control animals.

It is clear that the relationship between salt and water intake is an important factor in the toxicity of high NaCl. It is also evident that the two strains of mice used in this work are very different in their reactions. The resistant strain showed examples which lived over 500 days on salt when the normal life span is two years or more, while the susceptible strain showed no survivors over 90 days when the longevity is usually somewhat over a year. Four per cent salt is about the limit that can be tolerated in drinking water, which is above the concentration of sea water. The electrolytic conductivity equivalent of sea water is somewhat above 3% (14). Adolph (1) found that the survival of rats on sea water was 7–26 days, which was only slightly greater than when on no water at all (6–15 days).

The variation as to the susceptibility of littermates, some individuals dying in a relatively short time and others living for extended periods, is in keeping with the experience of others. The differences in tolerance between the two strains of mice are genetically determined. This aspect was studied by McNutt (9) by crossing these two strains and testing the F_1, F_2, F_3 and backcross generations for tolerance to 4% NaCl. His results suggested that the strains are not genetically homogeneous and that although the difference in tolerance for salt is not the result of a single gene, the number of genes is small.

The mice show interesting morphological changes when placed on high NaCl. Studies are now underway to determine the factors involved in the mechanism of the resistance or susceptibility to the deleterious effects of NaCl.

Summary and Conclusions

1. Two strains of mice were shown to differ in their resistance to the harmful effects of 4% NaCl administered in drinking water.

2. Weights of animals of both strains decline at first under salt treatment but those of the resistant strain (IHB) and the younger ones of the susceptible strain (NH) are able to adjust themselves for a time, and even regain some weight before shrinkage and death. Older animals of the susceptible strain are least able to maintain weight.

3. In the resistant strain, animals may survive as long as 510 days (17 months) on this regime, while in the susceptible strain none lived for 90 days (3 months).

4. As the age at which animals are placed on salt rises the length of survival decreases.

5. There is evidence that animals started on salt at less than 30 days of age often die within 10 days, but the ones that survive for 30 days behave like those initially placed on salt at 30 days.

6. There is a direct relationship between age and individual susceptibility to salt and this in turn is correlated with the relative longevity of the strain of mouse.

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SKELETAL MUSCLE STUDIES BY MEANS OF COMBINED ELECTROMYOGRAPHY AND NEEDLE BIOPSY[a]

I. THE ELECTROGRAPHIC MYOTOME

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Duchenne, inspired by the work of Griesinger and Bilroth in obtaining muscle tissue by means of a needle biopsy, fashioned his own instrument which he designated a tissue punch. (2) By means of this instrument, Duchenne obtained small samples of muscle which he examined in their fresh state and at that time described many of the microscopic characteristics of the childhood form of muscular dystrophy (pseudohypertrophic muscular dystrophy). During the 75 years following Duchenne's publication the needle biopsy procedure law fallow and it was only till Shank and Hoagland (3) described another myotome that a brief resurgence of interest occurred. Adams *et al.* (1) have criticized the needle biopsy procedure for study of skeletal muscle in that they feel that such instruments do not obtain adequate quantities of tissue.

Though recognizing the criticisms inherent in the needle biopsy procedure of studying muscle disease, a series of investigations have been initiated. This first publication in a series of studies on muscle disease is devoted to a description of a new needle biopsy instrument and the technic for using it. It is called the electrographic myotome because it incorporates the electromyographic electrodes and a cutting element into a single instrument. This combination makes it more than likely that an abnormal portion of tissue can be obtained if disease is present.

The electrographic myotome (Figure 1) consists of 4 basic

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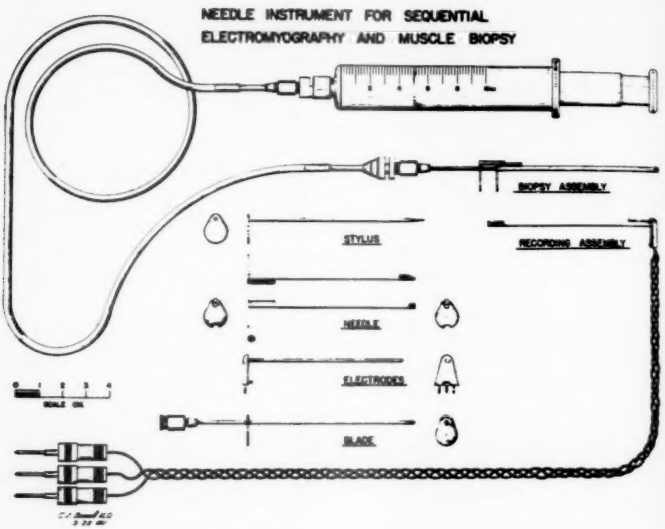

FIG. 1. The Electrographic Myotome.

elements; 1) an outer penetrating hollow needle, ground to a point with a window cut in its side, 2) a stylus which fits snugly into the penetrating needle, 3) a pair of shielded stainless steel electrodes, and 4) a hollow core cutting needle which fits snugly into the penetrating needle and has a luer connection at its proximal end to allow connecting with a hypodermic syringe for aspiration of tissue after it is cut. The penetrating needle varies from 14 to 18 gauge (outside dimensions) and is constructed from stainless steel needle stock tubing. The stylus is made from stainless steel rod. The electrodes are constructed from stainless steel wire (30–40 gauge) and are insulated from each other and the shield by means of a cold setting plastic (epoxy-resin).

The electrographic myotome is used in the following way: After a surgical scrub and sterilization of the skin with Merthiolate, the area over the muscle to be biopsied is infiltrated with procaine 1%. The area is draped with a sterile myotome towel and a stab wound through the skin 2 to 3 mm in length is made. The myotome with the stylus in place is passed through the skin incision and subcutaneous tissue then it is quickly plunged into

the muscle mass. The myotome is inserted at a right angle to the main muscle mass rather than parallel to the muscle fibers as was done by Shank and Hoagland.[3] When it is considered that the myotome is properly positioned in the muscle, the stylus is withdrawn and the electromyograph electrodes are inserted and positioned in the window of the penetrating needle. The patient is asked to flex his muscle and correctness of insertion is ascertained by examination of the electromyogram. After obtaining a photograph of the electromyogram trace on the oscilloscope, the electrodes are withdrawn. The cutting needle is connected via a flexible plastic tube to a 20 cc hypodermic syringe and is then inserted midway into the penetrating needle. A vacuum is induced in the cutting needle by drawing out the syringe plunger to the 7–10 cc mark. Immediately, following this maneuver while the vacuum is maintained, the cutting needle is slid rapidly to its final position in the penetrating needle and the whole assembly is quickly withdrawn from the muscle. Usually the bit of muscle remains in the tip of the cutting needle, however, on occasion it is aspirated into the plastic tubing or into the syringe itself. For this reason it is desirable to have previously rinsed the syringe and plastic tubing with sterile saline or Hank's solution. The specimen of tissue obtained is expelled aseptically into 5 to 10 cc of sterile Hank's solution in a Petri dish. Samples are then cut under aseptic conditions for preparation of histologic sections, for tissue culture and for the determination of specific gravity. Usually, sufficient muscle is obtained for all three purposes; the core of muscle obtained measures 4 to 6 mm in length and is approximately 1 mm in diameter.

The incision is closed by approximately the edges with adhesive tape. In over 120 biopsies performed during the past 12 months, no discomfort has been reported, no bleeding other than a few drops has occurred and no infections have been encountered. The procedure is recommended as safe and painless and as an accurate method of obtaining a biopsy from the diseased portion of a muscle. Furthermore, repeated biopsies from the same muscle or biopsy site can be performed at bi-monthly intervals with full patient co-operation. These advantages of the needle biopsy technic require emphasis since the proponents (1) of the grosser surgical procedure ignore such criticisms as follow: 1) the surgical biopsy often obtains only normal muscle despite

the size of the sample, 2) appreciable post-operative discomfort associated with scarring is the rule, 3) multiple biopsies are unreasonable from the viewpoint of patient discomfort and scarring. In this connection it can be stated that there is no satisfactory laboratory animal for the study of muscular dystrophy. The electrographic myotome together with a patient population as afforded by a clinic easily circumvents this aspect of the dystrophy problem. The investigator is thus assured that he is working with dystrophic muscle, and further, he knows the clinical type.

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WEIGHTS OF THYMUS AND SEMINAL VESICLE IN CASTRATE MICE AS ALTERED BY INTRAPERITONEAL VS. SUBCUTANEOUS INJECTIONS OF TESTOSTERONE*

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Introduction

Growth of isotransplants of adrenal cortical carcinomas of mice, when dependent upon castration of the host, was inhibited by subcutaneous administration of 7.5 mg of testosterone‡ every 20 days (Browning, 1958; Browning, White and Sadler, 1959). Growth of similar spontaneous tumors, in their original castrated hosts, was not affected by the same administration, even at 10 day intervals (Browning, Sadler and White, 1960). This lack of inhibition may have been due to an insufficient dose-level of steroid to affect spontaneous tumors, the smallest of which had a volume of 40 times that of an isotransplant. The present study was undertaken to estimate the dose-level of exogenous testosterone in the castrate that would be the biological equivalent of the endogenous androgen of the intact animal and to determine if modes of administration other than subcutaneous might be more effective.

Material and Methods

Three series of experiments were performed. In the first, 4 of 5 groups of 8 male A strain mice, 5 to 6 months of age, were castrated. They received, respectively, 0.0, 0.125, 0.25, or 0.375 mg of testesterone subcutaneously on the day after castration and 10 days later (Table 1, A-E). In the second, 3 of 4 groups of 8 male A strain mice, 3½ to 4 months of age, were castrated. They received, respectively, 1.0, 5.0, or 10.0 mg of testosterone subcutaneously at the same intervals as the animals in the first series (F-I). Fresh weights of seminal vesicles and thymus glands were obtained on the 20th day for all animals, including those in the untreated intact group of each series.

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‡ Unless otherwise specified, "testosterone" indicates a micro-crystalline aqueous suspension of testosterone phenylacetate ("Perandren", Ciba) containing 50 mg per ml.

In the third series, all animals in 9 groups of 8 male A strain mice, 3½ to 4 months of age, were castrated. Two groups received 0.5 mg of testosterone (intraperitoneally or subcutaneously) on the day of castration and 10, 20, and 30 days later (J, K). The remaining 7 groups received the same maintenance dosage (administered subcutaneously) but, in addition, 4 groups received 7.5 mg of testosterone on the day of castration and 20 days later—one intraperitoneally, one subcutaneously, one subcutaneously after dilution of the preparation with an equal volume of distilled water, and one subcutaneously but with one-quarter fractions of each dose being injected at separate sites (L-O). The other 3 groups received the same total dosage over the same period as the preceding 4 groups, but the individual doses contained 1.5 mg of testosterone and were administered every 4 days, commencing on the day of castration, on 10 occasions. This administration was made intraperitoneally in one group, subcutaneously in another, and subcutaneously after dilution with an equal volume of distilled water in the other (P-R).

Fresh weights for the seminal vesicles and thymus glands were obtained for all animals in the 9 groups on the 40th day after castration.

Results

The mean weights, and standard errors, for the seminal vesicles and thymus glands for all groups are given in Table 1 with the probabilities of differences between consecutives means in each series being due to chance. The mean weights for the first and second series are shown plotted against a log-dose scale in Figure 1 and those for the third series as a histogram in Figure 2.

Maintenance dose level (Figure 1, Table 1)

In the first series (A-E), seminal vesicle weight in castrates was increased, compared to that in untreated castrates, by 0.125 mg and again by 0.25 mg of testosterone to a high degree of significance ($P - <0.001$). A dose level of 0.375 mg did not produce a further significant weight increase; at this level, the weight was approaching that found in untreated intact animals, differing from it at the 5% level.

In the second series (F-I), there was a significant ($P—<0.05$) weight increase between the 1.0 and 5.0 mg but not between the 5.0 and 10.0 mg dose leevls. Further, the weight at the 1.0 mg level did not differ significantly from that found in the untreated intact animals (while that at the 5.0 and 10.0 mg levels differed to a highly significant degree).

In the first series, 0.125 and 0.25 mg of testosterone did not affect *thymic weight*, relative to that in untreated castrates, although 0.25 mg produced a significantly lower weight than 0.125

TABLE 1

Mean weights and standard errors of the seminal vesicles and thymus glands in castrated strain A mice receiving testosterone at different dose levels or by different methods, with corresponding figures for untreated intact mice, and with probability of significance of differences between consecutive means

	Series and group	Dose in mg	Freq. & no. of doses	Mode of adm.	Mean wts. and standard errors with probabilities* Sem. Ves.	(Prob.)	Thymus	(Prob.)
	First							
A	Cast.	0.0	.....	..	22.7± 0.9		37.3±4.2	
						<0.001		.....
B	Cast.	0.125	10d/x2	SC	94.3± 7.7		41.9±2.4	
						<0.001		<0.05
C	Cast.	0.25	10d/x2	SC	182.2±19.9		34.6±1.3	
						.....		<0.05
D	Cast.	0.375	10d/x2	SC	213.0±15.3		26.4±3.6	
E	Intact	0.0	.....	..	255.9± 9.2		23.0±1.3	
	Second							
F	Intact	0.0	.....	..	198.9±10.8		28.1±1.9	
						.....		<0.05
G	Cast.	1.0	10d/x2	SC	221.1±13.3		20.0±2.2	
						<0.05		<0.001
H	Cast.	5.0	10d/x2	SC	279.4±19.9		6.0±1.1	
						.....		.....
I	Cast.	10.0	10d/x2	SC	301.6±16.9		5.9±1.2	
	Third							
J	Cast.	0.5	10d/x4	IP	346.9±21.2		29.1±4.6	
						<0.01		<0.01
K	Cast.	0.5	10d/x4	SC	258.7±16.2		14.2±2.1	
						.....		.....
L**	Cast.	7.5	20d/x2	IP	445.9±26.6		6.6±1.8	
						.....		.....
M	Cast.	7.5	20d/x2	SC	385.1±27.3		9.4±2.4	
						.....		.....
N	Cast.	7.5	20d/x2	SCd	369.7±21.3		5.9±0.7	
						<0.05		.....
O	Cast.	7.5	20d/x2	SCf	313.1±15.3		7.2±1.3	
P	Cast.	1.5	4d/x10	IP	439.3±32.8		9.6±0.9	
						<0.05		.....
Q	Cast.	1.5	4d/x10	SC	333.8±22.3		9.6±2.1	
						.....		.....
R	Cast.	1.5	4d/x10	SCd	356.5±20.4		10.0±3.0	

(SCd—diluted; SCf—each dose in equal fractions injected at different sites.)

* Weights in milligrams.

** Dosage for groups L–R in addition to maintenance dosage of 0.5 mg every 10 days on 4 occasions.

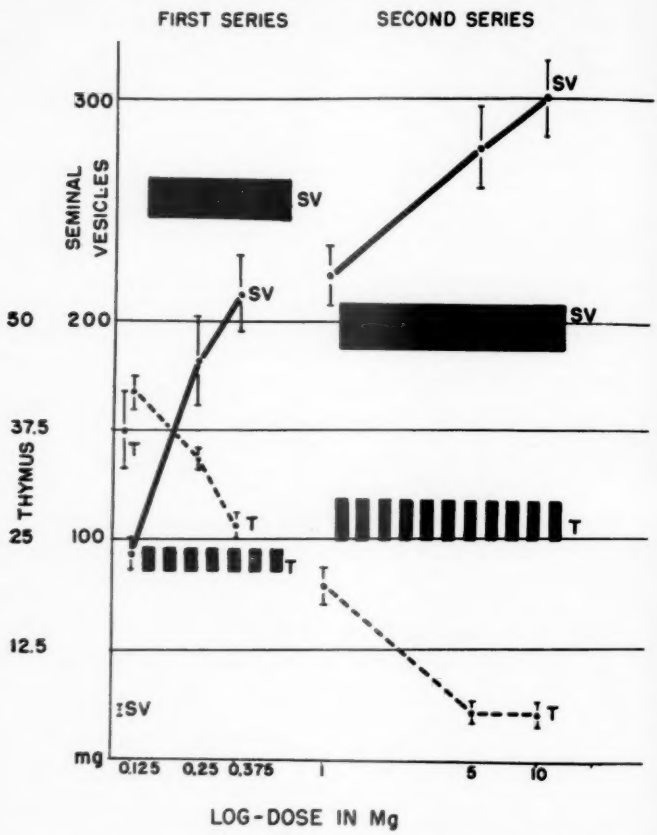

FIG. 1. Mean weights and standard errors of the seminal vesicles and thymus glands of two series of castrated A strain mice receiving various doses of testosterone subcutaneously. (Solid line—seminal vesicles; broken line—thymus glands; isolated point—untreated castrates; horizontal bar—untreated intacts).

mg, and 0.375 mg than 0.25 mg. This weight did not differ significantly from that in untreated intact animals at the 0.375 level (although it was greater, to a highly significant degree, at the 0.25 and 0.125 mg levels).

In the second series, thymic weight decreased to a highly significant degree, between the 1.0 and 5.0 mg but not between

the 5.0 and 10.0 mg dose levels. The weight was significantly lower than that in the untreated intact animals at the 1.0 mg (and highly significantly lower at the higher dose levels).

The dose of testosterone required to maintain the seminal vesicles and thymus glands of a castrate in a similar state to that in an intact animal evidently fell between 0.375 and 1.0 mg when administered subcutaneously at 10 day intervals for 20 days.

Seminal vesicle weight in untreated intact animals was higher, and thymic weight lower, in the first than in the second series. This is in accord with the fact that the animals concerned were 5 to 6 months, and 3½ to 4 months of age, respectively.

Mode of administration (Figure 2, Table 1)

From organ weights of the groups in the third series comparisons were made between intraperitoneal and subcutaneous administration; between administration subcutaneously, subcutaneously in dilution, and subcutaneously in fractions at different sites; and between administration in 2 doses of 7.5 mg or 10 doses of 1.5 mg.

At the 0.5 mg dose-level, *intrapertioneal* administration was more effective than *subcutaneous* in increasing seminal vesicle weight and less effective in decreasing thymic weight (J/K), both organs being heavier to a highly significant degree with the former. At the high dose-level (1.5 mg every 4 days: P-R), intraperitoneal administration again produced a higher seminal vesicle weight than subcutaneous (P—<0.05), or subcutaneous diluted (P—<0.05). With administration of 7.5 mg every 20 days (L—O), intraperitoneal administration produced a (non-significant) greater seminal vesicle weight than subcutaneous, a significantly greater (P—<0.05) weight than subcutaneous diluted, and a highly significantly greater (P—<0.001) weight than subcutaneous fractionated. In contrast, thymic weights were depressed to the same degree at the higher dose-level, regardless of the rout or mode of administration.

Dilution versus non-dilution of subcutaneous doses (either 1.5 or 7.5 mg, Q/R, M/N) produced no significant difference in the weights of the seminal vesicles, but fractionation gave significantly less effect upon the seminal vesicles in comparison with dilution (P—<0.05, N/O) or non-dilution (P—<0.05, M/O).

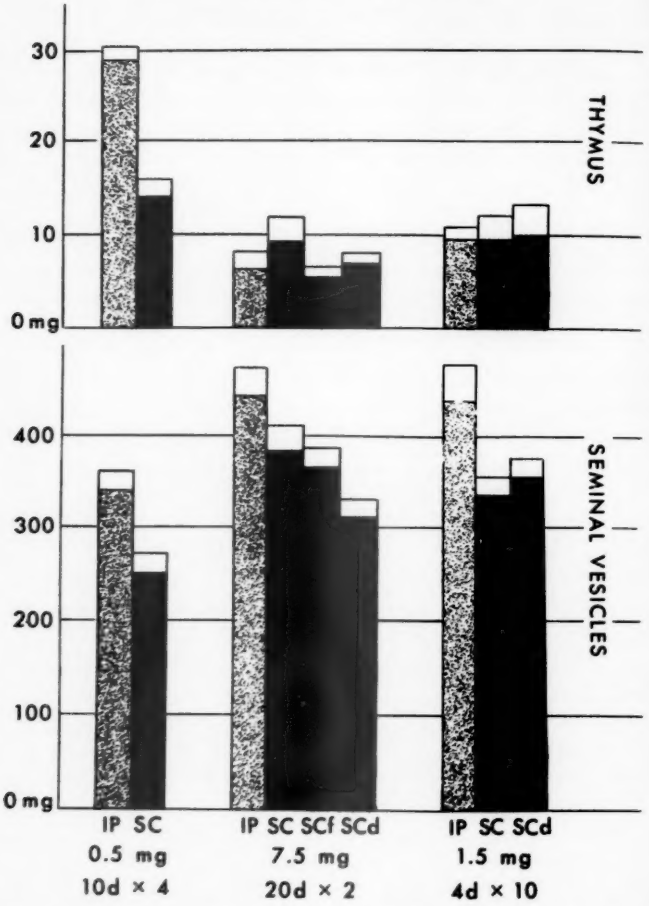

FIG. 2. Mean weights of seminal vesicles and thymus glands of a third series of castrated A strain mice receiving testosterone at two dose levels by various modes of administration. (Positive standard errors indicated by the blank area on each bar).

Administration of the same total amount of testosterone, in 10 doses rather than two, had no significantly different effect upon the seminal vesicles or thymus glands by the intraperitoneal (L/Q) route.

Discussion

Subcutaneous administration of 10.0 mg of testosterone every 10 days did not depress thymic weight more than the 5.0 mg dose; seminal vesicle weight was increased but not to a significant extent. Probably a maximal effect had been reached at the lower dose level; presumably it also had been in mice whose spontaneous adrenal tumor growth was not inhibited by 7.5 mg given subcutaneously at the same intervals (Browning, Sadler and White, 1960). However, intraperitoneal administration was always more effective than subcutaneous, at high or low dose levels in the present study, on the seminal vesicles although not on the thymus glands. The intraperitoneal rout might be more effective in inhibiting growth of spontaneous adrenal cortical tumors.

Intraperitoneally administered androgen is probably absorbed, in part via the hepatic portal system with some resultant inactivation by the liver (Biskind and Mark, 1939). Such inactivation could account for the lesser effectiveness of the androgen by this route on thymic weight at the lower dose levels. Simultaneously the intraperitoneal androgen might be absorbed directly by the seminal vesicles; such absorption could account for their greater responsiveness both at the higher and lower dose levels. If so, then injection into the superior mediastinum, avoiding hepatic portal and seminal vesicle absorption but allowing direct action upon the thymus, might reverse the differential effect at the lower dose levels. At the higher levels, presumably, a sufficient excess of androgen is present to compensate for hepatic inactivation, thus eliminating a differential effect upon the thymus gland but still allowing direct action upon the seminal vesicles. On the other hand, intraperitoneal administration may result, in contrast to subcutaneous, in the formation of metabolites that are more effective upon a sexual target organ (seminal vesicles) than upon a non-sexual one (thymus gland).

With this androgen (testosterone phenylacetate) of prolonged action (Kupperman, et al, 1954), there was no evidence of regression of the stimulated seminal vesicles, or of recovery of the involuted thymus gland, when the organ weights in animals receiving their last dose 10, rather than 4, days before autopsy were compared (groups L/P and M/Q, Table 2).

Testosterone proprionate, when administered intraperitoneally, had no effect upon the seminal vesicles of rats (Leathem, 1948) and was relatively ineffective on the capon comb at low dosages (Bernstorf, 1957). However, the androgen was administered in an oily vehicle in both cases.

Summary

1. The maintenance dose of testosterone phenylacetate, in castrated A strain mice, was between 0.375 and 1.0 mg when given subcutaneously every 10 days.

2. Intraperitoneal administration was more effective than subcutaneous at low and high dosages in increasing seminal vesicle weight; it was as effective in causing thymic involution at high dosages but less effective at low dosages.

3. The effectiveness of a high total does was the same over a period of 40 days, whether given at 2 or 10 equal intervals.

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INHIBITION OF UPTAKE OF L-LYSINE-U-C^{14} INTO CHROMATOGRAPHIC FRACTIONS OF CATIONIC NUCLEAR PROTEINS BY 5-BIS-(2'-CHLOROETHYL)-AMINOURACIL*

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Following the administration of ardioactive amino acids to tumor-bearing rats the nuclear proteins of the tumor were labeled to a greater extent that the nuclear proteins of other tissues (2,4,8). Recent studies have indicated the presence of a radioactive protein peak (RP2-L) in a variety of tumors which was not present in growing or nongrowing nontumor tissues following *in vivo* injection of L-lysine-U-C^{14} (9, 10). The administration of 5-bis-(2'chloroethyl)-aminouracil (11,14,15,16) to tumor-bearing rats resulted in a marked inhibition of incorporation of L-arginine-U-C^{14} into nuclear proteins of the tumor and spleen, but had virtually no effect on the incorporation of the labeled isotope into cytoplasmic proteins (1). Similar results for other mustards have recently been reported by Nyhan (17) who studied the effects of methyl-bis-(2-chloroethyl)-amine (NH_2) and the nitrogen mustard derivatives of alanine and phenylalanine on the incorporation of labeled amino acids into proteins of tissues in tumor-bearing rats. The development of methods for the chromatography of cationic nuclear proteins (9) provided the means for study of the specificity of the inhibitory effect of the uracil mustard on amino acid incorporation into the individual components of the histones. The present studies were designed to determine the effects of the aminouracil mustard on the incorporation of L-lysine-U-C^{14} into various chromatographic protein frations of the acid-soluble nuclear proteins of tissues of tumor-bearing rats.

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Materials and Methods

The Walker 256 carcinosarcoma was implanted 7 to 10 days prior to the experiment in male rats weighing 180–220 grams obtained from the Holtzman Rat Company (Houston). The mustard, 5-bis-(2′-chloroethyl)-aminouracil,[1] dissolved in dimethylformamide, was administered intraperitoneally as a single dose of 4 mg per kg to rats bearing the Walker tumor (1). At various time periods ranging from 6 to 24 hours after administration of the mustard, the rats were sacrificed. In each instance, 10 μc of L-lysine-U-C^{14} (Nuclear Chicago Corp.) with a specific activity of 12.9 mc/mmole was injected intraperitoneally 1 hour prior to sacrifice of the animal. The tissues were rapidly excised, placed in ice-cold 0.25 *M* sucrose, and homogenized in isotonic sucrose (1:9 w/v) in the cold room (4° C). The nuclei of tumor and liver were isolated by differential centrifugation (5). For the nuclear preparation of the spleen, the precipitate collected at 600 × gravity was utilized (18).

The cationic nuclear proteins were extracted from the nuclei by treatment with 0.25 *N* HCl for 30 minutes. The acid-extract was separated from the insoluble residue by centrifugation at 600 × gravity and clarified by centrifugation at 100.000 × gravity for 30 minutes. The supernatant solution was dialyzed overnight against 0.5 *N* acetic acid at 4° C and chromatographed in the cold room on carboxymethylcellulose columns as previously described (9) with 1 and 8 *N* formic acid as the eluting agent.

The protein concentration in the effluent fractions was measured by determination of the optical density of the solutions at 280 mμ in a Beckman DU spectrophotometer. Aliquots of each effluent fraction were pipetted onto stainless steel planchets, dried by warm air on a rotating turntable and assayed for radioactivity in a Nuclear-Chicago automatic counting system.

Results

Kinetics of incorporation of L-lysine-U-C^{14} into cationic nuclear proteins.—The patterns for protein distribution and radioactivity in the chromatograms of the cationic nuclear proteins of the Walker tumor, liver and spleen were the same in these studies as those reported previously (9). Figure 1 presents the specific activities (cpm/E_{280}) of the protein of the whole acid-extract and the chromatographically isolated fractions of the nuclear preparations of the Walker tumor, spleen and liver at various times after administration of the tracer. Maximal incorporation of the isotope in samples of tumor and spleen occurred at 1 hour while maximal incorporation of the isotope in samples of liver was found at 6 hours after administration of L-lysine-

[1] 5-bis-(2′-chloroethyl)-aminouracil was generously supplied by the Upjohn Company, Kalamazoo, Michigan, through the courtesy of Dr. Harold G. Petering.

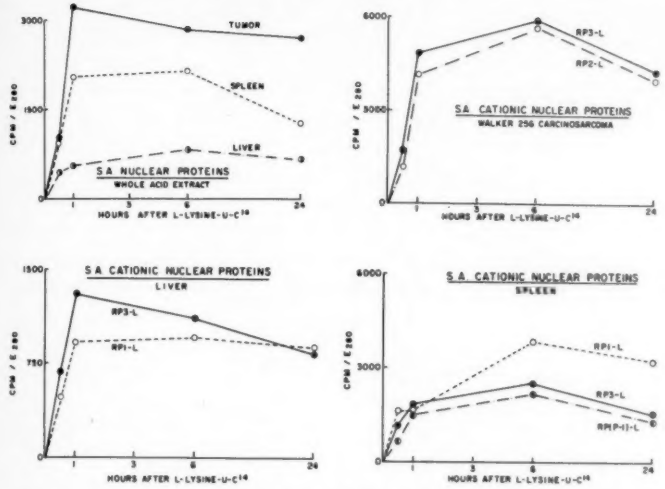

FIG. 1. Kinetics of incorporation of L-lysine-U-C¹⁴ into cationic nuclear proteins of the Walker 256 carcinosarcoma, liver and spleen. The data are the averages of 2 to 4 experiments. The ordinate is a linear scale and the abscissa is a logarithmic scale. The specific activity is counts per minute divided by optical density at 280 mμ.

U-C¹⁴. The kinetics of incorporation of L-lysine-U-C¹⁴ into various chromatographic fractions on the Walker tumor, liver and spleen differ in a number of respects from the kinetics of labeling of the whole acid extract (Figure 1). In the Walker tumor, maximal labeling of the proteins of RP2-L and RP3-L occurred 6 hours after administration of the tracer, followed by parallel decreases in labeling of these two fractions up to 24 hours after the tracer. In the liver, maximal labeling of the proteins of both RP1-L and RP3-L occurred 1 hour after administration of the tracer. In the spleen, the specific activity of the proteins of RP(P-1)-L, RP1-L and RP3-L each reached a maximum 6 hours after administration of the tracer. With the exception of the specific activities, the chromatograms were identical at each time point and RP2-L was not present in samples of liver and spleen at any time point studied.

Suppression of uptake of L-lysine-U-C¹⁴ into cationic nuclear proteins by 5-bis-(2'-chloroethyl)-aminouracil.—Inasmuch as the labeling of protein was approximately linear up to one hour

after injection of L-lysine-U-C^{14} (Figure 1), the optimal time for the study of drug effects was taken to be 1 hour after administration of the radioactive lysine. The patterns for protein distribution and radioactivity in the chromatograms of the cationic nuclear proteins following the administration of the aminouracil mustard were the same in these studies as previously described (9). Figure 2 presents the specific activities of the proteins of the whole acid-extract of the nuclear preparations of the Walker tumor, spleen and liver 6, 12, and 24 hours after administration of the aminouracil mustard. The aminouracil mustard suppressed labeling of proteins of the whole acid-extract of tumor to 65 and 20 per cent of control values at 6 and 24 hours after its administration and suppressed labeling of proteins of spleen to 40 and 22 per cent of control values at the same time. In liver, a two- to four-fold stimulation of amino acid incorporation into acid-soluble nuclear proteins was noted at 12 hours after administration of the drug.

The effects of the aminouracil mustard on the incorporation of L-lysine-U-C^{14} into the chromatographic fractions of the Walker tumor, liver and spleen are also presented in Figure 2. In the Walker tumor, the specific activity of RP2-L and RP3-L were both approximately 50 per cent of control values within 6 hours after the administration of the drug. At 24 hours after administration of the drug, the specific activity of RP2-L and RP3-L was 30 and 43 per cent, respectively, of control values. In the spleen, at 6 hours following the administration of the drug, the labeling of RP(P-1)-L and RP1-L was suppressed to 22 and 29 per cent, respectively, of control values, while the labeling of RP3-L was suppressed to only 50 per cent of control values. In the liver, a marked stimulation of the labeling of L-lysine-U-C^{14} into the proteins of both RP1-L and RP3-L occurred at 12 hours following the administration of the aminouracil mustard.

Table 1 summarizes the effects of the aminouracil mustard on labeling of the various chromatographic peaks of tumor, spleen and liver at 12 hours after the administration of the drug. The aminouracil mustard markedly suppressed the incorporation of L-lysine-U-C^{14} into both the whole histone and the chromatographic fractions of tumor and spleen but stimulated labeling of proteins in the liver. In addition, labeling of the protein peaks

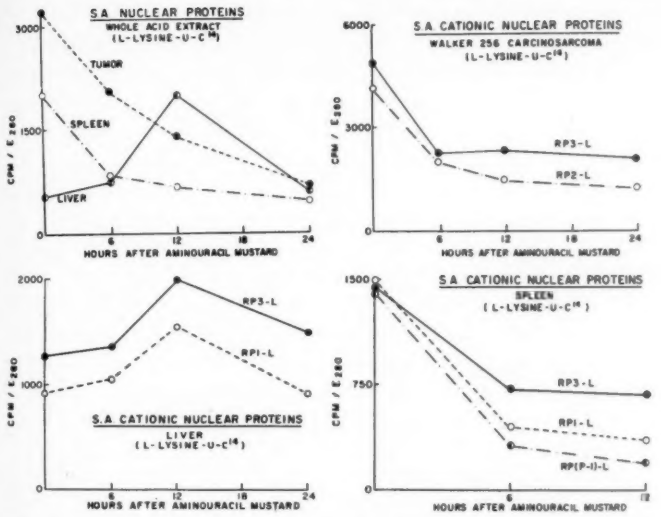

FIG. 2. Kinetics of the effects of the aminouracil mustard on incorporation of L-lysine-U-C¹⁴ into cationic nuclear proteins of the Walker 256 carcinosarcoma, liver and spleen. The data are averages of 2 to 4 experiments. The specific activity is counts per minute divided by optical density at 280 mμ.

TABLE 1

Per cent of Control Specific Activity in Chromatographic Peaks at 12 Hours After Administration of Aminouracil Mustard

	Tumor	Spleen	Liver
Whole Acid-Extract	42 ± 1.0	32 ± 1.0	365 ± 76
RP(P-1)-L	.....	.....	.....
RP1-L	.....	25 ± 5.0	173 ± 16
RP2-L	36 ± 3.0	.....	.....
RP3-L	49 ± 5.0	46 ± 1.0	153 ± 18

The data are expressed as average per cent of control values ± the standard error and represent averages of 2 to 7 experiments. The aminouracil mustard was administered in a dose of 4 mg per kilogram body weight.

eluted early was inhibited to a greater extent in both tumor and spleen than labeling of RP3-L which was eluted with 8 *N* formic acid.

Discussion

Previous reports in the literature have indicated that a major site of action of various mustards is the cell nucleus. These findings have, however, been based on morphological evidence such as nuclear fragmentation (12) and appearance of chromosomal bridges (7). Earlier studies from this laboratory have shown the marked inhibitory effects of the mustard on labeling of the nuclear proteins (1). The data obtained in the present study of the inhibitory effects of the uracil mustard on the incorporation of labeled lysine into the various chromatographic fractions of the cationic nuclear proteins have extended these observations and have emphasized the remarkable effect of this drug on the metabolism of the spleen (13). The present data indicate a partial tissue specificity of the aminouracil mustard in its effect on the spleen. Moreover, intracellular specificity of the action of the agent is present in that inhibition of labeling of the protein peaks RP(P-1)L and RP1-L was greater than inhibition of labeling of RP3-L which was eluted with 8 *N* formic acid. A similar result was found for the Walker tumor. Such a result represents an advance in chemotherapy, since it may be possible to devise drugs which exert more marked effect on RP2-L of the tumor than was found with aminouracil mustard and the various fractions of the spleen. Such data provide a continuing justification for attempts to produce selectivity of anti-tumor effects by means of structural modifications of alkylating agents.

The increased activity of the liver in incorporation of isotope into cationic nuclear proteins at 12 hours after administration of aminouracil mustard indicates that the liver is affected by the drug. The mechanism of this effect is not clear but may be a release of feedback controls on synthesis of ribonucleoproteins as the pre-existing ribonucleoproteins were alkylated by the mustard.

Another aspect of the present studies is the relative constancy of the chromatographic patterns of the cationic nuclear proteins of the tissues over a twenty-four hour period following injection of the labeled amino acid. RP2-L has been reported to be a distinguishing feature of chromatograms of a number of tumors (10). Since most of the early studies were carried out at one hour after administration of the tracer, the possibility existed that appearance of RP2-L might merely be delayed in other tissues. The

failure of RP2-L to appear in chromatograms of liver and spleen at any time point povides further support for the concept that its appearance is peculiar to chromatograms of the tumor.

Summary

At varying time periods ranging from 6 to 24 hours after pretreatment with 4 mg of 5-bis-(2'-chloroethyl)-aminouracil per kg, rats bearing the Walker 256 carcinosarcoma were injected intraperitoneally with 10 μc of L-lysine-U-C¹⁴. Studies on kinetics of labeling of cationic nuclear proteins with L-lysine-U-C¹⁴ indicated that labeling was linear up to one hour after administration of the tracer. Hence, one hour after injection of the tracer, the acid-soluble nuclear proteins of tumor, liver and spleen were isolated and chromatographed on carboxymethylcellulose. The aminouracil mustard suppressed labeling of proteins of the whole acid-extract of tumor to 65 and 20 per cent of control values at 6 and 24 hours after its administration and suppressed labeling of proteins of spleen to 40 and 22 per cent of control values at the same time. In liver, a two- to fourfold stimulation of amino acid incorporation into acid-soluble nuclear proteins was noted at 12 hours after the drug. C¹⁴ uptake into RP2-L of the tumor was suppressed to 49 and 30 per cent of control values at 6 and 24 hours, respectively, after drug administration. Studies on kinetics of labeling of nuclear proteins indicated that RP2-L was not found in spleen or liver at any of the time points up to 24 hours after injection of L-lysine-U-C¹⁴. The data indicate that the effects of the aminouracil mustard are in part specific, and provide a continuing justification for attempts to produce selectivity of anti-tumor effects by means of structural modifications of alkylating agents.

Acknowledgments

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ERYTHROCYTE SURVIVAL IN NORMAL AND STRESSED RATS*

C. E. Hall, J. B. Nash and O. Hall†

The hematologic effect of stress includes a reduction in circulating lymphocytes and eosinophils, an increase in neutrophils (3,7,12) and, in man, an apparent increase in circulating erythrocytes (4,13). The latter phenomenon, so called "stress polycythemia," appears to be due chiefly to a reduction in the plasma component of the blood. There is evidence indicating that lysis plays a part in the reduction of lymphocytes and eosinophils, although the sequenstration of these in areas outside the blood stream complicate the precise assessment of its magnitude. Such stress effects appear to be mediated largely through the agency of adrenal cortical hormones, since they do not occur after stress in patients with adrenal insufficiency. The ability of adrenal hormones to affect the erythrocyte composition of blood is indicated clinically by the anemia of Addison's disease and the polycythemia of Cushing's disease, respectively states of adrenal cortical insufficiency and excess, and experimentally in the rat by the significant elevation in erythrocyte, hemoglobin and hematocrit values which ensue upon prolonged treatment with hydrocortisone or corticosterone (6). The purpose of the present study was to determine whether any of the erythrocytic changes ascribed directly to stress or secondary to the hormonal changes induced thereby could be accounted for by a prologation in the life span of this cell.

Materials and Methods

Thirty-two female rats of the Holtzman strain weighing 110–120 grams were used in the study. Group 1 consisted of 20 animals which were stressed by placing them individually into cages, through the flooring of which brief shocks were delivered every two minutes for a consecutive 12 hours daily throughout the experiment. The method has been described in detail elsewhere (9) and its efficacy demonstrated (8,10). Group 2 consisted of twelve unstressed rats. Purina Laboratory Chow‡ and water were freely available

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‡ The Purina Co., St. Louis, Mo.

for 12 hours a day and unavailable for the remainder of the period to rats in both groups. A 1.25 ml volume of Rachromate, Cr^{51}, having a specific activity of 47.4 mc/mg and a concentration of 0.01 mg Cr/cc was incubated for 20 minutes at room temperature with 22.5 ml of blood obtained from the inferior vena cava of donor rats of the same strain, and 12 cc of Owen's ACD solution (disodium citrate 2.5%, citric acid 0.8% and dextrose 1.2%) at pH 5. Ascorbic acid, 5 mg in 0.5 ml of ACD solution, was then added to reduce unbound chromate and the mixture thoroughly shaken. Tagged blood, in a volume of 0.5 ml containing approximately 7 μc of Cr^{51}, was then injected into the tail vein of each of the recipient rats. Twenty-four hours later samples were drawn by heart puncture, weighed in a test tube on an analytical balance, and the volume calculated from the weight and known specific gravity (14) of blood. This served as the time reference (T_0) and allowed for the destruction of those tagged cells which were damaged by the manipulations incident to tagging and destroyed within 24 hours of infusion. Stressing was begun immediately after the T_0 sample was withdrawn. Additional blood samples were taken at weekly intervals, being obtained by severance of the tail tip, to measure disappearance of radioactive cells. Corrections were made for known decay, but not for elution of chromate from erythrocytes. On each occasion when blood was withdrawn for estimation of Cr^{51} activity, a sample was taken for a micro-hematocrit measurement.

Results

The decline in radioactivity of the blood in each of the groups is shown in Figure 1. It will be noted that with the possible exception of a slight difference between the groups in respect to

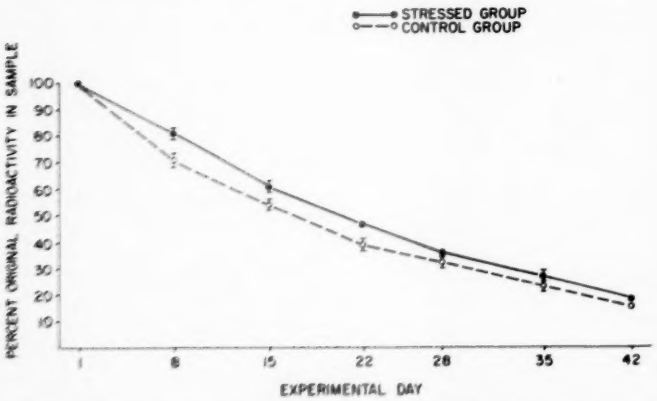

Fig. 1. Temporal decline in the radioactivity of blood from stressed and non-stressed rats. The mean half-life of the red cells and the rate of their disappearance from circulating blood was the same in the two groups.

radioactivity present in the sample taken at the first week, red cell survival was essentially identical in each group. Growth, and the hematocrit values of tail blood at each of the sample periods is given in Table 1. Although stress was severe enough to cause three animals in the exposed group to die during the experiment, and to cause marked impairment of growth in the survivors—terminal average body weights being significantly less ($P<.001$) in them—there was no detectable difference in the hematocrit nor in the overall survival of the red cells.

TABLE 1

Body Weight and Hematocrit of Stressed and Non-stressed Rats

Data		Group: Stress	Group: Control
No Rats			
	Initial	20	12
	Final	17	12
Body Wt. (grams)			
	Initial	117 ± 1*	118 ± 1
	Final	166 ± 4	189 ± 2
Hematocrit			
	0	45 ± 0.4	44 ± 0.6
	7	44 ± 0.5	44 ± 0.4
	14	47 ± 0.7	45 ± 0.5
Day			
	21	45 ± 0.4	45 ± 0.5
	28	45 ± 0.4	45 ± 0.4
	35	43 ± 0.3	45 ± 0.4

* Mean ± S.E. of Mean.

Discussion

In an earlier experiment we reported that the half life of red cells in the rat as determined by the Cr^{51} method was about 8 days (11). Other investigators at about the same time reported a like figure (1,5). However since then experiments have been reported indicating a half-life of 18–20 days (2,15). The present results are in accord with these later findings, the controls, in which two animals showed an unusually rapid destruction of transfused cells, showing a half-life of 17 days, and the stressed, a half-life of 20, and suggest that the shorter half-life obtained in the earlier experiments was due to the use of Cr^{51} having a low

specific activity, with consequent damage to the tagged erythrocytes by chromate ion. There is evidence to indicate that the manipulation of blood incident to the tagging procedure impairs the viability of many red cells which, as a consequence, disappear from the circulation within 24 hours of transfusion. Red cell survival is computed as the percentage of originally tagged cells remaining in the circulation at periods after their introduction. Therefore, the selection of T_0 as 1 hour after introduction of tagged cells, as in earlier experiments, rather than at 24 hours later as in the present instance, would also serve to shorten the apparent half-life.

Hemodynamic plethora or "stress polycythemia" (4) in man is a disorder of uncertain status. It is said to be more common in males, to be accompanied by an increased hematocrit and a normal red cell volume and to be due to a reduction in plasma volume precipitated by psychogenic stress (13). In the present experiment control animals never exhibited hematocrits greater than 48. In the third week three of the stressed animals had hematocrits greater than 50, but this was not sustained. Computation of the change in plasma volume from the formula: Fractional change in

$$\text{plasma volume} = \frac{\text{Plasma vol.}_2 - \text{Plasma vol.}_1}{\text{Plasma vol.}_1} = \frac{(\text{Hematocrit}_1 / \text{Hematocrit}_2) - 1}{1 - (\text{Hematocrit}_1 / 100)}$$

did not indicate that there was a significant difference between the plasma volumes of the stressed and the non-stressed rats at any time during the experiment. Thus there was no indication of polycythemia in stressed rats, either from the information provided by the hematocrit or from the calculations of residual radioactivity.

Summary

The results obtained indicate the half-life of the rat erythrocyte to be between 17 and 20 days. Chronic stress of the type used did not alter the red cell half-life, nor affect the hematocrit. Under the circumstances, stress neither produced the polycythemia said to be caused in man, nor that which hydrocortisone or corticosterone induce in the rat after lengthy treatment at high dosage.

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CARDIAC AND RENAL LESIONS DUE TO FLUDROCORTISONE AND FACTORS INFLUENCING THEIR DEVELOPMENT*

C. E. Hall and O. Hall†

Potent mineralocorticoid steroid hormones cause hypertensive cardiovascular disease in many mammalian species, including man. In the rat so affected, cardiac lesions are prominent. They consist of diffuse myocarditis or fibrosis; inflammatory granullomatous areas surrounding cardiac arteries or arterioles the walls of which show varying degrees of thickening, necrosis and/or hyalinization. There also occurs arteriolar nephrosclerosis and visceral periarteritis nodosa (4,5,10,11,12,15), all of which manifestations appear to be dependent upon the Na ion. Glucocorticoids, on the other hand, cause hypertension which is not dependent on NaCl intake, is not intensified by removal of a kidney, and does not cause vascular lesions in the rate (1,2,9). Curiously, glucocortcoids such as cortisone appear able to prevent the induction of periarteritis nodosa by mineralocorticoids such as desoxycorticosterone when both are administered together, but aggravate nephrosclerosis (14). There are thus circumstances in which mineralocorticoid and glucocorticoid activity may be either mutually antagonistic or reinforcing.

Synthetic 9-halogenated corticosteroids which exhibit both glucocorticoid and mineralocorticoid properties in high degree, also cause severe vascular lesions in the rat. It has been reported that 9-α-chloro-cortisol and 9-α-fluoro-cortisol (fludrocortisone) each causes hypertension and nephrosclerosis in the rat sensitized to steroid action by unilateral nephrectomy and a high NaCl diet, but neither was found to induce cardiac lesions and only the former caused periarteritis (13).

Recently we have had occasion to study some effects of fludrocortisone (6) and became intrigued with certain of its properties. In particular we wondered whether the apparent inability

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to cause cardiac lesions and periarteritis inidicated too little mineralocorticoid activity to evoke them, or a strong glucocorticoid activity which prevented them. The following studies were undertaken to gain further insight into the cardiovascular effects of the steroid. The first experiment was designed to evaluate differences in response between unilaterally nephrectomized and intact animals, and the second experiment to compare the pathophysiologic effects of restricted and unrestricted NaCl intake in animals sensitized to treatment by removal of a kidney.

Materials and Methods

In the first experiment 26 female rats of the Holtzman strain weighing 51–62 gm. were divided into three groups. Group 1 consisted of ten animals subjected to unilateral nephrectomy; groups 2 and 3 of ten and six intact animals respectively. All were given 1% NaCl drinking solution and Purina Laboratory Chow *ad lib.* The animals of groups 1 and 2 each received 250 μg/day of fludrocortisone acetate* as an aqueous microcrystalline suspension subcutaneously. The blood pressure of unanaesthetized animals was measured by a tail plethysmograph on the 21st day of the experiment. The survivors were killed with ether on the 28th day, and hearts, kidneys and pancreas were excised for weight and/or histologic examination.

In the second experiment twenty-nine female rats of the same strain, weighing 60–72 gm, were divided into three groups numbering 11, 10 and 8 respectively. All had one kidney removed and were allowed free access to Laboratory Chow. Rats of the first two groups each received 250 μg of fludrocortisone subcutaneously daily, and those of group 3 served as uninjected controls. Groups 1 and 3 were allowed unlimited intake of 1% NaCl drinking fluid, but each animal in group 2 from the second day on was allowed only that amount which had, on the average, been consumed by the untreated animals in group 3 on the preceding day. Blood pressures were measured on the 16th day and survivors were killed on the 20th. Just prior to sacrifice a sample of tail blood was collected from each rat for a microhematocrit. At autopsy the heart, kidneys, adrenal glands and a sample of pancreas were taken for weight and/or histology. Lesions in heart, kidney and pancreas were noted. Severity was arbitrarily graded on a 0 to +++ scale and expressed as a percentage of the theoretical maximum in both experiments.

Results

It was evident in the first experiment that steroid injected, unilaterally nephrectomized rats were much more severely damaged by treatment than were intact rats. There were no deaths in groups 2 or 3 from treatment, but in group 1 deaths began to

* The steroid was generously supplied by E. R. Squibb and Sons.

occur on the 15th day, and by the 28th day 60% of the group had succumbed. All of these were found to have singly or in various combinations: plerual effusion, edema, ascites and gastrointestinal ulcers, and each had kidneys which were grossly flecked with small whitish specks.

On the 21st day of the experiment the average blood pressure was higher in group 1, where four of the five animals had pressures exceeding 150 mm Hg, than in group 2 where only two of ten animals exhibited such pressures. No hypertension was observed among controls. Steroidal impairment of body growth to about the same degree occurred in each of the two treated groups.

At autopsy no edema, ascites or pleural effusion was seen in any of the animals in groups 2 and 3. Two of the 4 survivors in group 1, however had extremely severe edema and ascites and another one of them also had moderate pleural effusion. Three had grossly detectable nephrosclerosis.

Histologic examination of the hearts and kidneys of group 2 and 3 animals revealed no detectable abnormalities, although in group 2 one animal had a kidney in which many tubules were filled with hyaline casts. In group 1 on the other hand, all kidneys examined, including those from animals which had died during the experiment, showed severe glomerulosclerosis or glomerular hyalinization. Most glomeruli in the sections were affected to a greater or lesser degree and there was the associated tubular atrophy and tubular cast formation typical of nephrons subjected to anoxic necrosis. No evidence of arterial or arteriolar necrosis or hyalinization was seen in the kidney, the lesion seemingly being restricted to the glomerulus. In none of these animals, however, was any vascular damage or myocarditis observed in heart sections, nor was periarteritis ever noted.

The heart weight of each steroid-treated group was significantly ($P<0.001$) enlarged to an equivalent degree over control values. The right kidneys of intact steroid-treated animals proportionately to body weight were also significantly larger ($P<0.001$) than those of untreated controls, averaging 37% greater weight. Those in group 1 animals having undergone in addition compensatory hypertrophy, were, of course, larger still. The data are given in Table 1.

In the second, as in the first experiment, the steroid caused inhibition of body growth, most marked in those on limited fluid

TABLE 1

Comparison of the Effect of Fludrocortisone on Intact and Uniephrectomized Rats

Data		Group 1	Group 2	Group 3
No. Animals	Initial	10	10	6
	Final	4	10	6
Body wt. g.	Initial	55 ± 1*	55 ± 1	56 ± 2
	Final	124 ± 17	118 ± 4	156 ± 4
Mean Blood Pressure mm Hg.	Day 21	146 ± 14	127 ± 6	118 ± 4
Heart	mg/100 g.	449 ± 19	465 ± 9	335 ± 7
Right Kidney	mg/100 g.	1392 ± 137	823 ± 15	602 ± 16
Vascular Lesions				
Heart	% Incidence	0	10	0
	% Severity	0	3.3	0
Kidney**	% Incidence	100	10	0
	% Severity	92	3.3	0
Periarteritis				
	% Incidence***	0	0	0

* Mean ± S.E. of Mean.
** Right kidney only.
*** In pancreas.

intake. Blood pressure, taken on the 16th day, revealed that 3 of the first group and none of the second were hypertensive. One of the controls was found to have a blood pressure of 146 mm. Hg; considered to be hypertensive by some laboratories, but "pre-hypertensive" in ours.

Mortality among group 1 was high, one animal died on the 19th day and three on the 20th day which prompted the decision to terminate the experiment then. Prior to death, edema, ascites and pleural effusion had developed in five of the ten animals in group 1 although these were never observed in either of the other groups.

Measurements made just prior to sacrifice, by taking micro samples from the tail of unanesthetized animals, revealed that the hematocrit was the same in groups 1 (45–53) and 2 (46–52) in both being greater than in group 3 (41–44), indicating hemoconcentration in the first two groups.

Microscopic examination revealed no evidence of periarteritis nodosa in the pancreas. Severe glomerular sclerosis or hyalinization was evident in every one of the group 1 animals, both those

which died and those that were killed (Figure 1). In addition all but one of them had severe cardiac lesions (Figure 2). One animal in group 2 and two of the controls, given only salt, however, were found to have identical but less severe renal and cardiac lesions (Figures 3 and 4). However, these were also, at autopsy, noted to have had hydronephrosis of the remaining kidney.

Cardiac hypertrophy was evident in group 1 animals, but not in those of group 2. Furthermore the kidney enlargement, largely compensatory hypertrophy, was of equal magnitude in groups 2 and 3, but in those with added renal lesions, group 1, the kidneys were of much larger size. The data are summarized in Table 2.

TABLE 2

Influence of NaCl Consumption on Untreated and Fludrocortisone-Treated Rats

Data		Group 1	Group 2	Group 3
No. Rats	Initial	11	10	8
	Final	7	10	8
Body wt. g.	Initial	68 ± 2*	66 ± 1	66 ± 1
	Final	94 ± 4	80 ± 2	138 ± 3
Blood Pressure mm Hg.	Day 16	140 ± 6	118 ± 3	122 ± 6
Hematocrit (terminal)		45–53	46–52	41–44
Organ wts.				
Adrenals	mg	24.2 ± 3.1	16.1 ± 1.2	46.9 ± 2.5
	mg/100 g.	25.3 ± 3.4	19.9 ± 1.3	34.0 ± 1.6
Heart	mg	454 ± 20	304 ± 11	549 ± 30
	mg/100 g.	496 ± 33	379 ± 7	399 ± 21
Left†	mg	427 ± 2	453 ± 10	431 ± 13
Kidney	mg/100 g.	623 ± 15	680 ± 19	650 ± 15
Right‡	g	1.43 ± 0.1	0.83 ± 0.03	1.37 ± 0.1
	g/100 g.	1.60 ± 0.1	1.03 ± 0.02	1.00 ± 0.1
Vascular Lesions				
Heart	% Incidence	91	10	25
	% Severity	48	4	13
Kidney	% Incidence	100	10	25
	% Severity	88	7	13
Periarteritis				
	% Incidence***	0	0	0

* Mean ± S.E. of Mean.
† At nephrectomy.
‡ At autopsy.
*** Pancreatic.

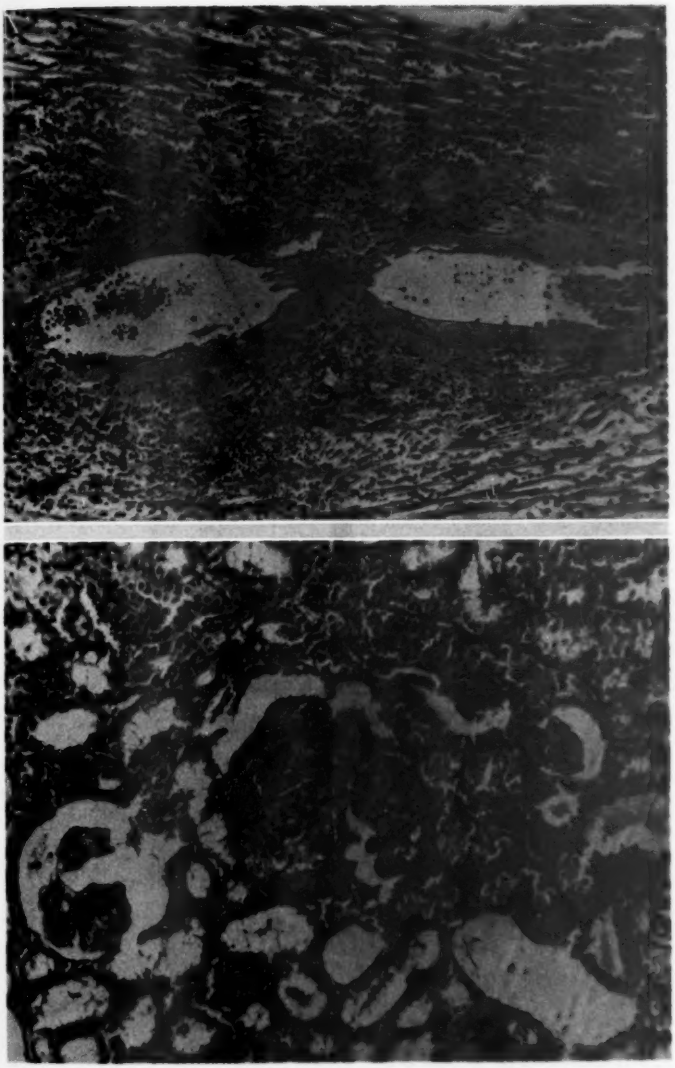

FIG. 1. The enlarged central glomerulus, from a fludrocortisone-treated rat, shows diffuse hyalinization. The one to its right appears shrunken. Many dilated tubules appear in the upper half of the section. H. and E. 140 ×.

FIG. 2. A large artery in the ventricular myocardium of a fludrocortisone-treated rat. The walls are completely hyalinized. There is an intense perivascular inflammatory infiltration extending radially into the ventricular muscle. H. and E. 119 ×.

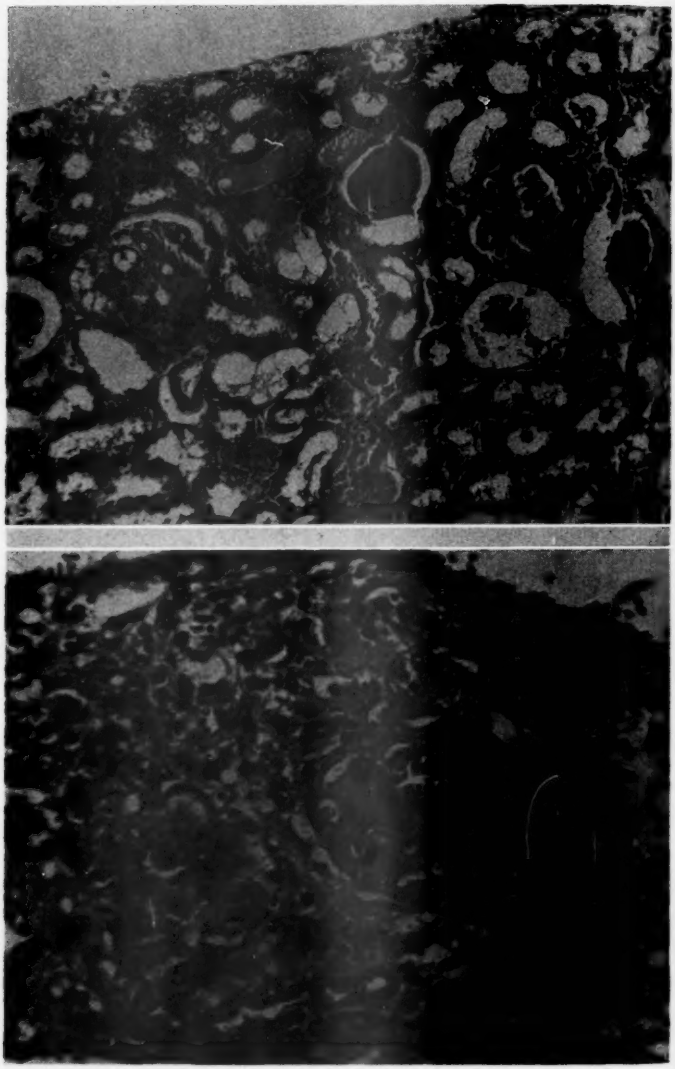

FIG. 3. Two severely hyalinized glomeruli in the kidney of a hydronephrotic salt-treated rat. Hyaline casts are present in some tubular lumens. H. and E. 140 ×.

FIG. 4. Cardiac artery from a hydronephrotic salt-treated rat showing a thickened and hyalinized wall. The adjacent tissue shows degeneration of muscle cells and infiltration by inflammatory cells. H. and E. 336 ×.

Discussion

From the second experiment it is quite clear that fludrocortisone possesses the ability to cause severe cardiac lesions when the circumstances are optimal. Its failure to do so in the first experiment in which the dosage was identical and steroid treatment was even more protracted and in others reported in the literature (13) is inexplicable, but is probably not attributable to the suppressive effects of inherent glucocorticoid potency, and clearly is not due to intrinsic lack of those mineralocorticoid properties necessary to induce cardiac damage.

In neither experiment were cardiac lesions ever seen in fludrocortisone-treated animals that did not also have moderate to severe glomerulosclerosis or glomerular hyalinization, and yet such glomerular lesions were often prominent and severe in animals without cardiac changes. It would seem, then, that the kidney is more easily damaged than the heart; so that by the time cardiac lesions appear, the kidney is already severely injured. Lesions in the heart, usually less diffuse and numerous than in the kidney, are also more apt to be missed unless serial sections are examined. Hypertension was not established as a necessary determinant of renal or cardiac lesions developed by fludrocortisone treatment, for although these were always found in animals with elevated blood pressure, they were often equally prominent in animals without. In the first experiment cardiac hypertrophy, usually regarded as a reliable index of elevated peripheral resistance was equivalent in both steroid treated groups even though those with intact kidneys had a lesser incidence and severity of hypertension.

It is recognized of course that unless blood pressures are estimated daily, transient episodes of hypertension might well be missed. Similarly, it has been established that hypertension severe enough to cause severe vascular lesions, may as a consequence of these abate to normal, near normal or even subnormal levels (3). Such lowered pressures are the result of vascular damage and cannot be etiologically related thereto.

The importance of salt and the amount of renal excretory tissue available for its handling appears clear-cut. Animals with intact kidneys withstood steroid treatment and sodium loading which produced high mortality, severe anasarca, extensive car-

dio-renal vascular lesions and hypertension in animals with only one kidney, and not only escaped these but developed only moderate hypertension in a few instances. On the other hand, as illustrated by Experiment 2, steroid-treated unilaterally nephrectomized animals on limited NaCl intake, escaped these pathophysiologic aberrations, provided that they were not hydronephrotic. It is thus evident that when the intake of 1% NaCl solution was restricted, the steroid did not cause sufficient retention of the cation to induce pathologic fluid shifts and vascular lesions therewith associated, even though NaCl intake was well above what would have been ingested with a normal diet. This focuses attention on the primacy of the electrolyte in the observed changes, in agreement with Ingle's (7) concept. However it is of interest to note that the relative hemoconcentration indicated by hematocrit values, was of similar magnitude in both restricted and unrestricted groups under steroid-treatment, although the former lacked either the anasarca or the hypertension so common among the latter.

Two control animals, one of which was normotensive (134 mm Hg) and the other "pre-hypertensive" (146 mm Hg), showed renal and cardiac lesions. Although such lesions are well know to be caused by NaCl excess, as we and others (8) have noted, the speed of their onset in the present instance undoubtedly was due to the fact that hydronephrosis had occurred in the only kidney. The lesions were identical in type to those developed under steroid-salt treatment, although somewhat less severe. Both of these animals had however a normal hematocrit, in contrast to the elevated hematocrit found in steroid-treated rats with similar lesions.

Adrenal involution was evident in both groups of steroid-treated animals. However, in those given unlimited salt the glands were significantly ($P = 0.02$) larger on an absolute basis than in those with limited intake, being in fact intermediate between those and the controls. The difference was not significant on a body-weight basis, but the true body weight is difficult to assess in animals so visibly afflicted with severe anasarca as were many of the rats on unlimited intake. The difference may have been due to the degree of stress caused by the extensive pathologic changes in non-restricted rats.

Cardiac hypertrophy, a good criterion of hypertension, was

present only in the group with unrestricted NaCl intake. Limitation of NaCl intake not only prevented cardiac hypertrophy, but also renal enlargement. The right kidneys of control rats with unrestricted NaCl intake, and those of fludrocortisone-treated rats with restricted intake, were enlarged, on a body weight basis, by 53% and 51% respectively over the left kidneys removed 20 days earlier. Those of fludrocortisone-treated animals with unrestricted intake were, on the same basis, 257% larger. The enlargement of kidneys under the circumstances obtaining, reflected the growth of these organs which normally would have occurred, even if uninephrectomy had not been performed, the compensatory hypertrophy consequent to removal of one kidney, and the changes in mass associated with the induction of renal lesions. Salt restriction undoubtedly eliminated the latter component, but may also have affected the others.

It may be concluded that fludrocortisone does inherently possess a mineralocorticoid potency sufficient to cause cardiac lesions, provide suitable conditions exist, and that their absence in certain treated animals probably does not reflect suppression of inherent glucocorticoid activity. The absence of visceral periateritis may be due as Seyle (14) proposed to the inhibition by glucocorticoid activity. On the other hand the development of periarteritis requires that the arterial pressure be moderately elevated for many weeks or greatly elevated for at least many days. In neither of these experiments, nor in those reported in the literature, does it appear that either of these requirements has been established. The failure to develop periarteritis may simply reflect the fact that pressure has not been sufficiently elevated for a long enough period.

Summary

Intact rats subjected to fludrocortisone treatment and a high NaCl diet for two or three weeks developed a low incidence of hypertension, were free of renal lesions and did not demonstrate subcutaneous edema, pleural effusion or ascites, all of which were prominent manifestations in similarly treated unilaterally nephrectomized animals.

In steriod-treated unilaterally nephrectomized animals, where such changes were otherwise severe and cardiac lesions were common, they could be prevented by simply restricting fluid and salt intake to the quantity voluntarily consumed by un-

treated controls. The hematocrit, however, was elevated in both steroid-treated groups to a comparable degree. Adrenal involution from steroid was marked when salt intake was restricted. Renal and cardiac lesions were not confined to animals with known hypertension. Limitation of NaCl to steroid-treated uninephrectomized animals, which greatly reduced cardiovascular and renal lesions, prevented the enhanced renal enlargement characteristic of those with unlimited intake.

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THE INFLUENCE OF HYPOTHERMIA ON THORACIC DUCT LYMPH

L. M. JOSEPH[b] AND S. N. KOLMEN[a, c]

Introduction

The influence of lowered body temperature on the physiological state of animals is of considerable interest. Many attempts have been made to use hypothermia as a therapeutic regimen (35) or to alter the status of the patient so that other therapeutic techniques may be implemented (3). Occasionally these attempts have resulted in complications such as intestinal hemorrhage (13, 28, 34), shock (6), and ventricular fibrillation (4, 10, 18, 38). It has been suggested that the complications are secondary to the effect of hypothermia on blood since proteins (1, 2, 7, 11, 32, 36), leucocytes (20, 21, 27, 35, 39, 40), 0_2 tension (5, 33), and pH (14, 16, 31) are altered in this state.

Another body fluid, lymph, may similarly be modified by the lowering of body temperature. It is the purpose of this paper to compare the effects of hypothermia on proteins and leucocytes in both blood and thoracic duct lymph.

Methods

1. *Surgical Procedure*

Mongrel dogs were used without regard to age, sex, or weight. All animals were allowed water *ad libitum*, but were deprived of food for sixteen hours prior to surgery. These animals, which weighed an average of 13 kilograms, were anesthetized with intravenous injections of 6% sodium pentobarbital (30 mg. per kg. body weight). A three-inch midline incision was made on the ventral surface of the neck extending superiorly from the suprasternal notch. The sternocleidomastoid and sternohyoid muscles were separated in the midline and the trachea was exposed. A tracheotomy was performed and a brass cannula of appropriate size was inserted.

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The carotid sheath was freed of surrounding fascia and displaced laterally. The thoracic duct was isolated as it crossed the suprascapular artery at a position immediately lateral and posterior to the suprasternal notch. Under fasting conditions the thoracic duct appeared as a glistening pink structure in the midst of surrounding fat. The duct was ligated near its entrance to the ampulla under the brachiocephalic vein and a second ligature was placed loosely about two centimeters below the ligation. Iris scissors were used to nick the thoracic duct and a polyethylene tube (Clay-Adams, PE 190) was rapidly inserted for several centimeters into the duct. The second ligature was then tied to prevent accidental removal of the thoracic duct catheter (23). The catheter was attached to a drop recorder.

Both femoral veins and one femoral artery were exposed surgically. A polyethylene tube (Clay-Adams, PE 205) was inserted into a slit in the right femoral vein and the catheter was threaded to the level of the thorax. The tube was then connected to a Statham strain gauge for the purpose of monitoring the venous pressure. The other femoral vein was cannulated so that blood samples could be obtained with comparative ease. The femoral artery was cannulated and attached to a Statham strain gauge for arterial pressure recordings on a Grass polygraph. The esophagus was exposed above the suprasternal notch and an incision was made just large enough to allow the passage of a thermister probe into the lower third of the esophagus. A second probe was inserted into the rectum of each animal. The temperatures from both sites were monitored by the use of a Yellow Springs Tele-thermometer (Model 46) to which the probes were attached.

Positive pressure breathing at a rate of ten strokes per minute was administered by means of a Harvard respiration pump attached to the tracheal cannula. Both respiratory rate and ventilatory volume were kept constant throughout the experiment. After the surgical procedures were completed, the arterial pressure, venous pressure, esophageal and rectal temperatures, and lymphatic flow rates were recorded. Samples of blood and lymph were taken at intervals from the zero time (the time immediately after surgery and completion of cannulations but prior to the institution of techniques which served to maintain or alter the body temperature).

2. *Temperature Regulation Procedure*

The deep core temperature of each of the eight control (normothermic) dogs was maintained within 0.1° C. of the temperature observed at zero time by the intermittent application of a tungsten filament heat lamp about two feet from the body surface.

Each of fifteen test (hypothermic) dogs was placed in a tub of crushed ice and water. Ice was placed around the portions of the ventral surface that were not immersed, care being taken to maintain the head above the level of the slush. The esophageal temperature was lowered approximately 10° C. in ninety minutes. Shivering was prevented by regulation of the depth of anesthesia.

3. *White Blood Cell Count*

White blood cells in citrated venous blood and thoracic duct lymph samples were counted on a Sahli-Adams-Neubauer cell counting chamber (24).

4. *Total Protein Assay*

Total proteins of venous blood and thoracic duct lymph were determined by the Biuret test for dipeptide linkages (41). The optical density was determined with a B & L "Spectronic 20" colorimeter at a wave length of 540 mμ. The protein standard used in all serum protein determinations was a 7.3 gm. per cent horse serum standard supplied by Cappel Laboratories, West Chester, Pennsylvania.

5. *Assays of Albumin and Globulin Concentrations*

Analysis of the albumin and globulin fractions in serum protein and thoracic duct lymph was carried out as specified in the Spinco Electrophoresis Manual (37). The paper strips were stained with bromphenol blue, and then the intensity of color was plotted on the Spinco Analytrol. The total area was divided into the albumin, alpha-1 globulin, alpha-2 globulin, beta globulin, and gamma globulin components. The percentage of the total area occupied by each component was integrated and recorded.

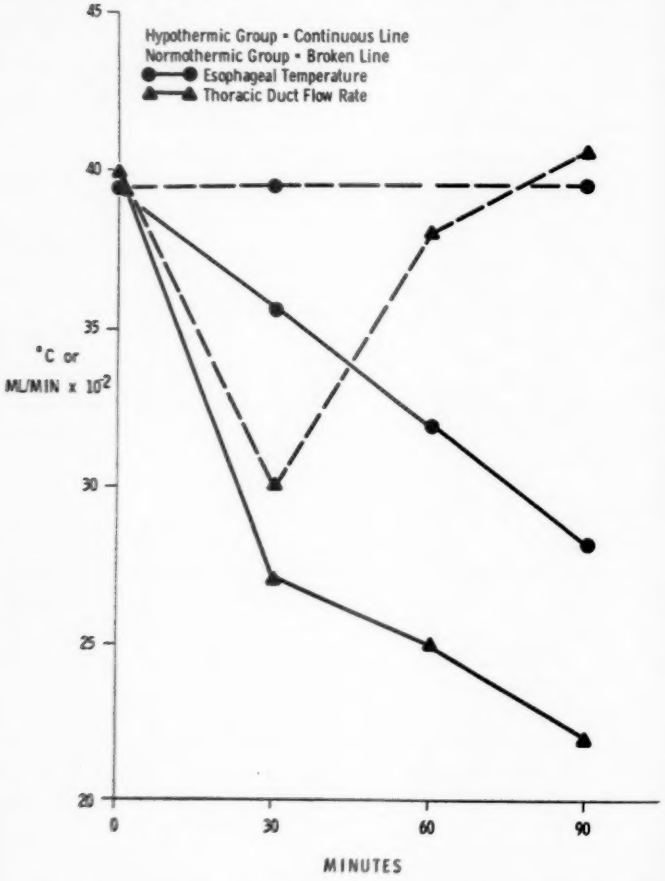
Results

Changes in body temperature over a ninety-minute period are depicted in Figure 1. The mean temperature of the normothermic animals was 38.5° C. although the zero-time temperatures ranged from 40.0° C. to 36.3° C. Under normothermic conditions it was noted that the body temperature of each animal did not vary over 0.1° C. throughout the ninety-minute test period. The mean zero-time temperature of the hypothermic group of dogs was also 38.5° C. When exposed to an icy environment the body temperature of the dogs decreased at the rate of 6.9° C. per hour (Figure 1). At the end of ninety minutes, the mean temperature of the hypothermic group was 28.1° C.

Since respiratory movements profoundly influence lymph flow rates, it was decided to maintain a constant ventilatory rate and volume in each dog with a respiratory pump. Blood pressures and heart rates, used as an index of the influence of hypothermia on the cardiovascular system, are averaged in Figure 2. The heart rate of hypothermic animals decreased gradually. At the end of ninety minutes, the rate of this group was about 50% of the zero-time value. The normothermic animals exhibited relatively constant systolic and diastolic pressures; and although a slight change in pulse pressure occurred in the hypothermic dogs, a gradual decline in both systolic and diastolic pressures was recorded. In no instance did the blood pressures of the hypothermic animals fall to shock levels. The 20% decrease in recorded pres-

FIGURE 1

BODY TEMPERATURE AND THORACIC DUCT FLOW RATE STUDIES



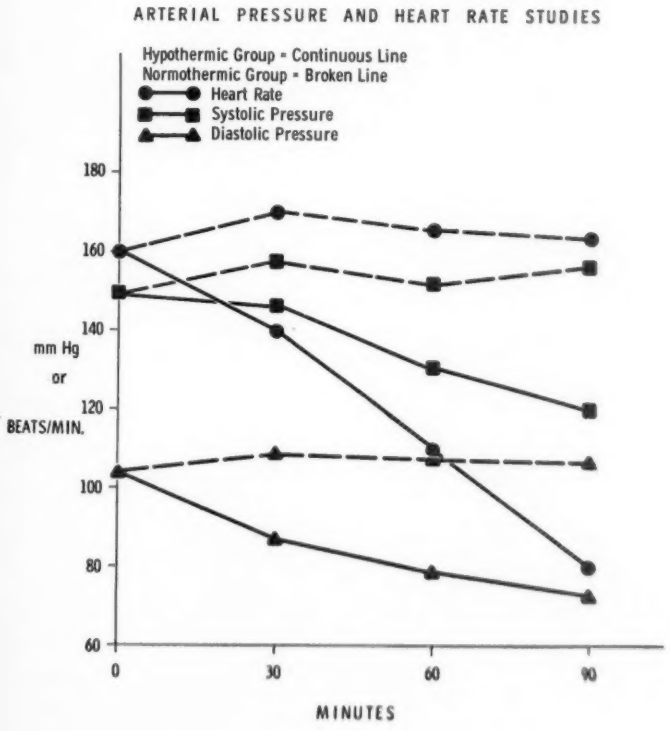
sures was within the normal variation of zero-time pressures and hence is not considered to have changed significantly.

The lymph flow rate (Figure 1) as recorded from the thoracic duct fistula characteristically changed with time in the normothermic dogs. There was a greater decrease in flow rate during the first thirty minutes of the experiment in both the normother-

mic and hypothermic groups than during the final sixty minutes. The initial decrease of 25–30% was followed by a return to zero-time flow rates in the normothermic animals whereas the hypothermic group exhibited a further decrease to 45% of the zero-time values.

Total protein concentrations of lymph and blood were determined by the Biuret test for dipeptide linkages. The mean value at zero time for total blood proteins was 6.9 ± 0.8 gm. per 100 ml. serum, while the mean value of total protein of lymph at zero time was 3.7 ± 0.2 gm. per 100 ml. serum. In Figure 3 the percentage deviation from these mean values is plotted against the time that the samples were obtained. Under normothermic

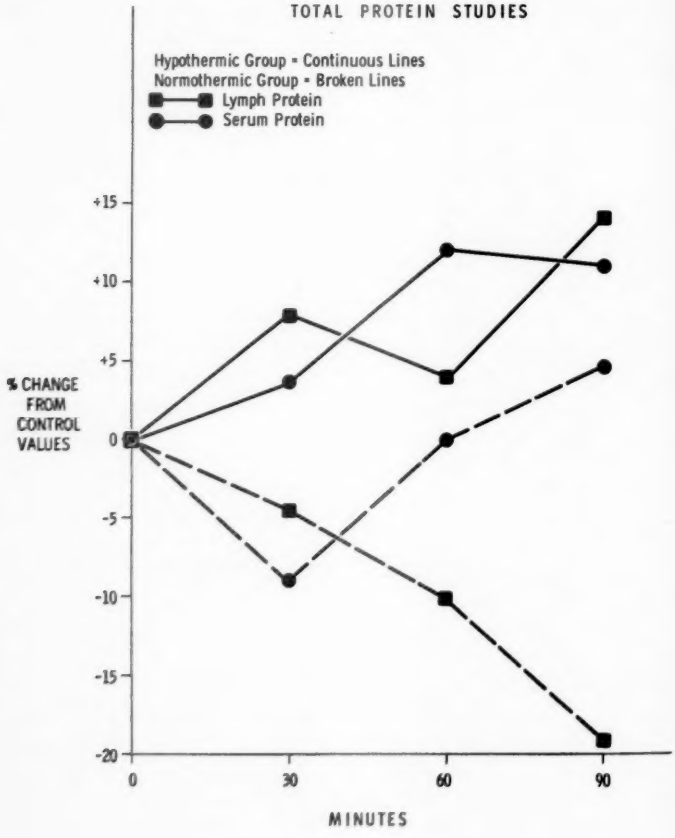
FIGURE 2



conditions the protein concentration in lymph decreased linearly so that by the end of ninety minutes there was a 19% decrease from the values at zero time. The protein concentration in the blood of these animals decreased in the first thirty minutes and thereafter rose to a concentration slightly higher than that recorded at zero time. Although initial protein levels were quite variable the above results were observed in all animals.

Under hypothermic conditions the total protein concentration

FIGURE 3



in blood and lymph increased by 11% and 19% respectively. The increase in proteins at the end of ninety minutes was considered to be significant ($p<0.1>0.05$) although the mean of samples obtained prior to this time were considered to be insignificant ($p<0.5>0.1$). The rise depicted in Figure 3 was consistently observed in this group of dogs.

An attempt was made to determine whether changes in the individual components of lymph and serum protein occurred. To measure the concentrations of the various protein components of the lymph and serum, aliquots were subjected to paper electrophoretic separations and analysis. The results, tabulated in Tables 1–5, are expressed as the percentage of albumin, alpha-1 globulin, alpha-2 globulin, beta globulin, and gamma globulin in these fluids. The albumin concentration (Table 1) of serum and lymph from both groups of dogs varied within normal limits. The apparent decrease in the concentration of albumin in the normothermic group also was within the limits of normal variation during the experimental period. Under hypothermic conditions more than 50% of the dogs exhibited an increase in both lymph and plasma albumin but again, this change was not statistically significant ($p\geq0.5$).

The changes in concentration of the various globulin fractions in both groups (Tables 2–5) were small and not consistent in either direction. Statistical evaluation of the data, using the two-tailed T-test for probability, indicated that all variations noted in the tables could have occurred by chance ($p\geq0.5$). From the data it therefore appears that major changes in the composition of lymph or serum proteins did not occur in either the normothermic or hypothermic groups.

TABLE 1

Albumin Concentrations During Experimental Period

Time, Minutes	Lymph		Serum	
	Normothermic	Hypothermic	Normothermic	Hypothermic
0	36.8[a]	36.8[b]	38.3[a]	38.3[b]
30	36.6	37.5	37.7	38.6
60	36.4	37.8	35.7	38.9
90	36.2	38.9	33.1	39.6

[a] The mean percentage of total protein obtained from 8 dogs.
[b] The mean percentage of total protein obtained from 15 dogs. Comparison of normothermic and hypothermic animals ($p \geqq 0.5$).

TABLE 2

Alpha-1 Globulin Concentrations During Experimental Period

Time, Minutes	Lymph		Serum	
	Normothermic	Hypothermic	Normothermic	Hypothermic
0	4.8[a]	4.8[b]	5.5[a]	5.5[b]
30	4.4	4.8	5.7	5.6
60	4.2	5.1	6.3	6.3
90	4.8	4.9	5.6	5.6

[a] The mean percentage of total protein obtained from 8 dogs.
[b] The mean percentage of total protein obtained from 15 dogs. Comparison of normothermic and hypothermic animals ($p \geqq 0.5$).

TABLE 3

Alpha-2 Globulin Concentrations During Experimental Period

Time, Minutes	Lymph		Serum	
	Normothermic	Hypothermic	Normothermic	Hypothermic
0	9.8[a]	9.8[b]	15.6[a]	15.6[b]
30	9.8	10.0	16.5	14.7
60	10.7	10.0	16.6	15.0
90	10.1	10.2	16.2	14.5

[a] The mean percentage of total protein obtained from 8 dogs.
[b] The mean percentage of total protein obtained from 15 dogs. Comparison of normothermic and hypothermic animals ($p \geqq 0.5$).

TABLE 4

Beta Globulin Concentrations During Experimental Period

Time, Minutes	Lymph		Serum	
	Normothermic	Hypothermic	Normothermic	Hypothermic
0	32.1[a]	32.1[b]	30.7[a]	30.7[b]
30	30.4	31.0	30.4	31.0
60	27.8	31.5	27.8	31.5
90	30.5	31.0	30.5	31.0

[a] The mean percentage of total protein obtained from 8 dogs.
[b] The mean percentage of total protein obtained from 15 dogs. Comparison of normothermic and hypothermic animals ($p \geqq 0.5$).

Analyses were also performed to ascertain whether lowering of the body temperature modified the white cell count of lymph or blood. White cells were chosen for the study because they are known to be present in both fluid compartments and they are suspended, rather than solubilized, in these media.

TABLE 5

Gamma Globulin Concentrations During Experimental Period

Time, Minutes	Lymph		Serum	
	Normothermic	Hypothermic	Normothermic	Hypothermic
0	11.2[a]	11.2[b]	11.9[a]	11.9[b]
30	9.4	11.1	12.9	11.7
60	11.0	11.1	12.1	11.4
90	10.0	11.5	11.5	11.6

[a] The mean percentage of total protein obtained from 8 dogs.
[b] The mean percentage of total protein obtained from 15 dogs. Comparison of normothermic and hypothermic animals ($p \geqq 0.5$).

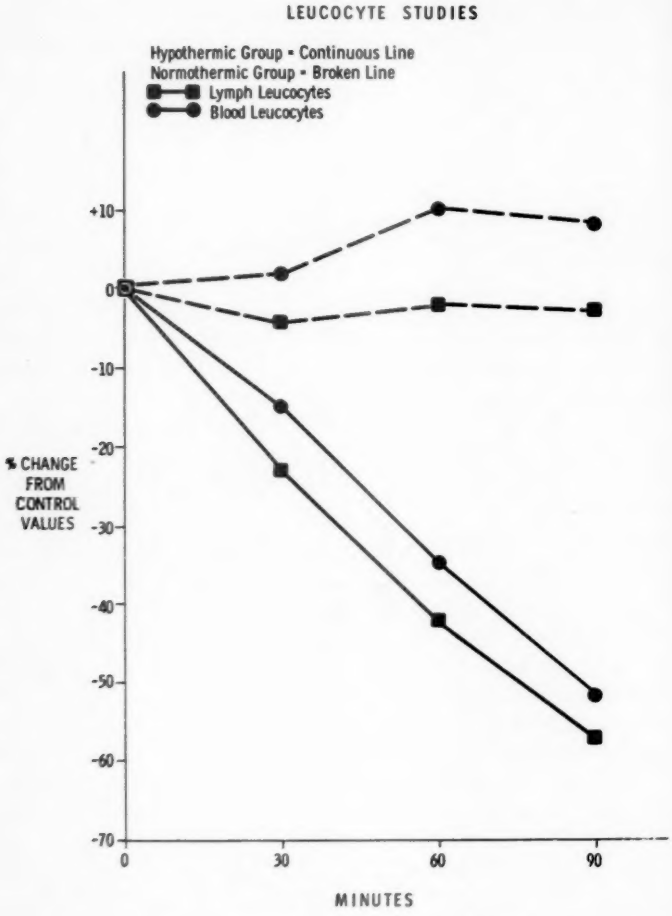
In Figure 4, the time at which the samples were collected is plotted against the percentage change of the white cell counts from those obtained at zero time. It will be noted that the white cell counts of lymph and blood taken from the normothermic group of dogs remained within 10% of control values at zero time. In contrast, the white cell counts of the hypothermic dogs decreased rapidly and in a fairly linear fashion; by the time the body temperature had decreased 10.4° C., the white cell count decreased 58% in lymph and 51% in blood.

Discussion

Hypothermic conditions were instituted successfully in fifteen anesthetized dogs of varied ages, sexes, and weights. Both rectal and esophageal temperatures were recorded; but on the basis of personal observations and those of Cooper and Kenyon (9), the esophageal temperature was considered to approximate more closely the deep core temperature. The rapid decline in body temperature (6.9° C. per hour) was made possible through the effects of deep pentobarbital anesthesia, namely, the abolition of shivering and the diminution of skeletal muscle tone. Respiration was maintained at a constant rate and volume so that the heat loss was continued through this avenue as well. The linearity of the drop in body temperature indicates that temperature-compensating mechanisms were inadequate to maintain body temperature under the experimental conditions employed.

Many factors influence the rate of thoratic duct lymph flow (42). Among these are (1) the "milking" action of skeletal muscle, respiration, and arterial pulsations; (2) capillary per-

FIGURE 4



meability and pressure; and (3) gastrointestinal absorption and villus movements. The level of anesthesia employed tended to abolish or effectively reduce most of these influences on lymph flow rate. Vasomotor tone, under the influence of the hypothalmic heat-conservation center (17), also, was diminished by the use of the above dosage of pentobarbital.

The maintenance of the rate and volume of respiration in animals subjected to a depression of body temperature may have resulted in a state of relative hyperventilation with a consequent hypocapnea. This hypocapneic condition and the peripheral vasodilation secondary to removal of central hypothalamic influences theoretically would result in a marked decrease in vasoconstrictor tone of arterioles. Some direct effect on the capillary endothelium may also have occurred.

Because of these experimental conditions, it was not surprising to find that the zero-time flow rates (Figure 1) of deeply anesthetized dogs were 50% of the lymph flow rates observed by other investigators (8). The mean flow rate was 0.4 ml./minute at zero time. It was observed that there was a comparable decrease in flow rates of both the normothermic and hypothermic animals during the initial thirty-minute period. This observation is indicative of influences which were not temperature dependent but were time dependent. The nature of these influences is not readily apparent. Perhaps these changes are a reflection of the effects of the surgical procedure and the initial depth of anesthesia. It is believed the changes that occurred after the initial decline are a reflection of temperatures to which the two groups were subjected. The subjection of normothermic animals to external heat in the maintenance of their zero-time temperatures may have been responsible for the slight increase in heart rate (Figure 2) and blood pressure which, in turn, would be expected to enhance lymph flow. The hypothermic group continued to show a diminution of flow rate which may have been secondary to the cardiovascular changes (Figure 2), to changes in the distribution of fluids from the extracellular spaces to the intracellular spaces (2), or to the decrease in intestinal or hepatic metabolism as a result of the temperature decline.

The changes in fluid compartments were not specifically investigated; however, the data in Figure 3 may present indirect evidence in favor of the shift of fluids from the vascular and lymphatic spaces into the intracellular space. In Figure 3, it was noted that the mean levels of total protein in both lymph and serum rose above those at zero time. Such a rise could be caused by either a loss of fluid without a comparable loss of proteins or an increased release of proteins from sites of storage. An increased release of protein due to *de novo* synthesis is unlikely

under conditions of lowered body temperature. An increased release of proteins bound to storage sites, also, is unlikely since the process of desorption or chemical unbonding is depressed by lowered temperatures (30). The above deductions in addition to the observations of Barbour, McKay, and Griffith (2), tend to support the hypothesis that fluids left the extracellular compartments and therefore lymph volume and flow rate decreased proportionately.

Changes in heart rate and blood pressure during these hypothermic studies essentially corroborated the observations of others (12, 19, 22, 29). The degree of bradycardia observed is consistent with findings of other investigators (12, 22, 29) and probably is a reflection of the effect of cold on the sinus node *per sé* since neither vagotomy nor atropinization alters this response (29). The heart rates of the normothermic animals were within normal limits at zero time. The slight increase in heart rate during the experiment may be attributed to the method of maintaining the dogs in the normothermic state.

The blood pressure of dogs subjected to hypothermia has been reported to increase initially and then to decrease at lower temperatures (29). The initial response was attributed to autonomic reflexes and the latter response to a decrease in cardiac output. The data presented in Figure 2 are suggestive that the level of anesthesia was sufficient to diminish autonomic reflex activity since no increase in pressures was noted. The fall in blood pressure probably is due primarily to the reduction in cardiac output since the peripheral resistance of both the systemic (5) and pulmonary circulations (25) is increased under hypothermic conditions.

From the circulatory standpoint, the observed changes in heart rate and blood pressure do not indicate the development of shock. On the other hand, a decrease in both heart rate and blood pressure results in a decreased contribution by the "milking" action of arterial pulsations and the hydrostatic pressure on lymphatic flow dynamics.

The increase in serum protein concentration of hypothermic animals has been amply elucidated (1, 2, 7, 11, 32, 36); however, studies related to thoracic duct protein concentration in hypothermia have been meager. McCarrell (26) noted that there was an increase in the lymph protein concentration of cervical lymph

but thoracic duct levels were variable in response to depressed body temperatures. In the present study there was a rise in protein concentrations of both the blood and the thoracic duct lymph of dogs subjected to hypothermia. Such an increase is in agreement with the previous suggestion (2) that there is a fluid shift into the intracellular compartment during hypothermia.

Any change in the total protein fraction of lymph or blood does not appear to be caused by a marked alteration in one component relative to the other protein components. When the total proteins were subjected to electrophoretic analysis, as noted in Tables 1–5, there were no significant changes of any fraction of protein. On the other hand, trends did occur although they were not statistically significant. The albumin fraction progressively increased in the lymph and serum of the hypothermic dogs. This observation has also been reported by Bober *et al.* (7), who observed significant increases in the alpha and beta globulin fractions with decreases in the gamma globulin fraction. However, the data presented here fail to indicate any change in the globulins. Thus, considering all available information, it appears that the increase in total proteins secondary to hypothermia was not due to an increase in any one particular fraction but of each fraction nonselectively.

The white cell counts of both blood and lymph taken from the normothermic dogs remained within 10% of the zero-time control values ($p \geq 0.5$). The lack of alteration of the leucocyte levels was also observed by Helmsworth, Stiles, and Elstun (20). The leucocyte count in both fluids decreased in a linear fashion when animals were exposed to a linear decrease in body temperature. Two possible mechanisms which might account for the decrease in white count with a lowered body temperature are: (1) a reduced rate of synthesis or (2) an increased rate of sequestration through adherence to the vascular intima. Using microcirculatory and histologic techniques, Miller and Eliot (27) observed an increased number of white cells adherent to the capillary walls of animals in the hypothermic state. Villalobos *et al.* (39) and Miller and Eliot (27) observed that the white cells and platelets in blood returned to normal levels on rewarming the dogs; the liver and the spleen were the primary sites of sequestration. The behavior of leucocytes in lymph during hypothermia has not been subjected to direct observation. Possibly the same phenom-

enon of sequestration is also present in the lymphatic compartment.

The influence that the adrenal cortex may exert in hypothermia is not fully understood. Perhaps the leucopenia observed in lymph and blood was the result of activity of the hypophyseal-adrenal cortical axis. However, Egdahl, Nelson, and Hume (15) found that the 17-hydroxycorticosteroid output in the adrenal vein fell to 25% of normal when the body temperature of pentobarbital-treated dogs was lowered to 25° C. The ACTH secretion also was depressed but an infusion of additional ACTH did not alter cortical secretion. For these reasons, it is not believed that the observed reduction in leucocytes is a reflection of stimulation of the hypophyseal-adrenal cortical axis.

Summary

Hypothermic conditions resulted in the reduction in lymph flow from the thoracic duct and an increase in the total protein concentration of both lymph and serum in dogs. The increase in proteins may be attributed either to the release of stored proteins or to the shift of solvent from the extracellular spaces into the intracellular compartment. The latter appears to be the more plausible explanation. There were no significant alterations in the individual electrophoretic fractions of the total proteins, and therefore, the hypothermic technique employed does not appear to be an adequate means to produce a deficiency of a specific protein fraction. The leucocyte counts of both lymph and blood samples declined linearly with the decline in temperature. Evidence is cited indicating that the leucopenia in blood is the result of cellular sequestration along the vascular intima. The mechanism for the reduction of leucocytes in lymph remains to be elucidated.

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BIOLOGICAL ACTIVITIES OF SOME HOMOLOGS OF ISOLEUCINE AND VALINE*

MAUNG CHIT MAUNG, CHARLES G. SKINNER AND WILLIAM SHIVE†

Mutual antagonisms among natural amino acids having similarities in structure have been known for some time (11), and there have been numerous reports of antagonisms among leucine, isoleucine and valine (3,4,5,7,8,14). From the structural viewpoint, valine and isoleucine are closely related to one another, the latter compound being a higher homolog of the former.

In the present investigation, a number of higher homologs of isoleucine were prepared in an effort to demonstrate the specificity of the α-alkyl grouping with regard to ability to compete with the natural substrates, isoleucine and valine, for essential enzymes. Since unsaturated α-amino acids frequently possess antimetabolite activity, 2-amino-3-methyl-4-hexenoic acid was also prepared and its biological properties were determined.

The biological properties of the analogs were determined with various microorganisms including specific nutritional mutants of *Escherichia coli* 9723 requiring either DL-valine or DL-isoleucine. Although the analogs did not inhibit the growth of several microorganisms including an isoleucineless *E. coli* 9723, they are relatively potent inhibitors of the utilization of valine in a valineless mutant of *E. coli* 9723.

EXPERIMENTAL[1]

Biological Assays[2]

The microbial assays utilizing *Lactobacillus arabinosus* 17-5, *Streptococcus faecalis* 8043 and *Streptococcus lactis* 8039 were carried out in a previously

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[1] All melting points are uncorrected. A Perkin-Elmer model 154B was utilized for the vapor phase chromatographic studies using a Perkin-Elmer column C packed with silicone oil, and the determinations were carried out at 140° under 15 lbs./sq. in. pressure. The paper chromatographic data were determined by the ascending technique in the indicated solvent pairs, and

described amino acid medium (15) which was modified by decreasing the DL-isoleucine and DL-valine concentrations to 10 μg./ml. each, and which contained 0.2 μg./ml. of calcium pantothenate. For *Leuconostoc dextranicum* 8086, the modified media described above was also used except that 0.02 μg./ml. of pantethine was added, and the phosphate salts concentration was increased four-fold. All of these assays were incubated at 30° for about 18 hours.

For the assays with *Escherichia coli* 9723, as well as the isoleucineless and valineless mutant strains, an inorganic salts-glucose medium was employed (2). This medium for the latter two organisms was supplemented with 10 μg./ml. of DL-isoleucine and 10 μg./ml. of valine, respectively, to induce near maximal growth. These assay procedures have been reported in detail (9).

Organic Syntheses

The commercially available alkyl halides, 3-bromopentane, 2-bromopentane, 3-bromohexane, and 2-bromohexane, were redistilled and characterized by physical techniques prior to being used in this study; n^{20}_{D} were 1.4450, 1.4416, 1.4475, and 1.4450, respectively. The literature values reported are 1.4443 (16), 1.4415 (16), 1.4450 (17), and 1.4432 (10), respectively.

4-Bromo-2-pentene

From a reaction mixture of 20 g. of 3-pentene-2-ol, 5 ml. of pyridine, and 12 ml. of phosphorus tribromide there was ultimately recovered 15 g. of lachrymatory product, b.p. 50–51° (60 mm); 120–123° (740 mm.); n^{20}_{D} 1.4660. Reported values are b.p. 72° (145 mm.) and 116.5–119.5° (760 mm.) (13).

Alkylation of Ethyl Acetamidocyanoacetate

The interactions of the various alkyl halides with ethyl acetamidocyanoacetate were all carried out in essentially the same manner with yields varying from 20 to 30%. The data on the individual derivatives are presented in Table 1, and the actual conditions for the condensation of 3-bromohexane are described as a typical preparative procedure. To a reaction mixture containing 2 g. of sodium in 150 ml. of ethanol there was added 15 g. of ethyl acetamidocyanoacetate, and, after solution was effected, 15 g. of 3-bromohexane was added slowly with stirring. The reaction mixture was heated under reflux for about 4 days during which time the pH of the solution became neutral to wet litmus paper. After cooling, the salts which precipi-

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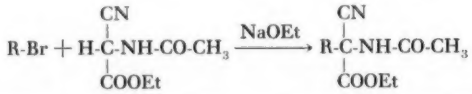
all the spots were developed with ninhydrin reagent. The authors are indebted to Dr. J. M. Ravel and Mrs. J. Humphries for assistance with the microbial assays, and to C. Hedgcoth and J. D. Glass for the elemental analyses.

[2] Nutritional mutants of *E. coli* 9723 deficient for valine, isoleucine and isoleucine-valine were isolated using a procedure comparable to the penicillin method as modified by Gorini and Kaufman (12). The authors are indebted to Mr. Thomas W. Conway, The University of Texas, Austin, who, through an efficient modified procedure, isolated and characterized these nutritional mutants.

TABLE 1

Some Substituted Ethyl Acetamidocyanoacetates



R	M.p., °C.	Empirical Formula	Analysis					
			Calculated Per Cent			Found Per Cent		
			Carbon	Hydrogen	Nitrogen	Carbon	Hydrogen	Nitrogen
2-Pentyl-	108–109	$C_{12}H_{20}N_2O_3$	59.95	8.39	11.66	60.12	8.50	11.92
3-Pentyl-	74–75	$C_{12}H_{20}N_2O_3$	59.95	8.39	11.66	59.77	8.28	11.71
2-Hexyl-	99–100	$C_{13}H_{22}N_2O_3$	61.37	8.72	11.02	61.25	8.72	11.25
3-Hexyl-	69–70	$C_{13}H_{22}N_2O_3$	61.37	8.72	11.02	61.02	8.60	10.78
4-(2-Pentenyl)-	109–110	$C_{12}H_{18}N_2O_3$	60.46	7.61	11.76	60.55	8.07	11.86

tated were separated and the solvent was removed *in vacuo*. The residue was treated with ethyl acetate, the insoluble material was filtered, and Skellysolve B was added to the clear solution to induce a slight turbidity. After standing at about 0° overnight, a product was recovered which, after recrystallization from ethanol-water, melted at 69–70° dec. The analytical data are recorded in Table 1.

Hydrolysis and Decarboxylation of Substituted Ethyl Acetamidocyanoacetates

All of these reactions were carried out in a similar fashion, and the data for the individual α-amino acids are recorded in Table 2. The specific conditions for the preparation of 2-amino-3-ethylhexanoic acid are described as a typical procedure. A mixture of 3 g. of ethyl 2-acetamido-2-cyano-3-ethylhexanoate in 25 ml. of concentrated hydrochloric acid was heated under reflux for 8 hours. The resulting reaction mixture was treated with Darco G-60, filtered, and the solution was concentrated *in vacuo* to dryness. The residue was extracted with three 10 ml. portions of ethanol; the extract was concentrated *in vacuo* to dryness. The residue was extracted with water, treated with about 10 g. of Dowex-3 anion exchange resin in the hydroxyl form, and allowed to stand overnight at room temperature. After the resin was separated and the solvent was removed, the residue was crystallized from ethanol-water to yield about 1 g. of 2-amino-3-ethylhexanoic acid, m.p. 234–236° dec. The analytical data are recorded in Table 2.

The R_f values for the α-amino acids synthesized in this study are presented in Table 3.

2-Amino-3-methyl-4-hexenoic Acid

A mixture of 4 g. of ethyl 2-acetamido-2-cyano-3-methyl-4-hexenoate and 25 g. of barium hydroxide in 30 ml. of water was heated under reflux in a stainless steel beaker for about 5 days. The insoluble matter was removed and the filtrate was then treated with carbon dioxide. After filtration, the solution was adjusted to pH 5 with 5% sulfuric acid to remove the final traces of barium ions. The latter filtrate was reduced to a small volume *in vacuo* and placed in a refrigerator overnight. There was recovered 1.6

TABLE 3

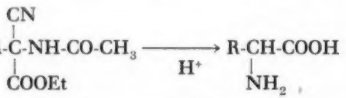
R_f Values of Some α-Amino Acid Analogs

Amino Acid	Chromatographic Solvent*		
	95% Methanol	Butanol:acetic acid:water (4:1:1)	65% Pyridine
2-Amino-3-ethylpentanoic Acid	0.75	0.69	0.65
2-Amino-3-methylhexanoic Acid	0.78	0.76	0.68
2-Amino-3-ethylhexanoic Acid	0.83	0.79	0.75
2-Amino-3-methylheptanoic Acid	0.86	0.94	0.85
2-Amino-3-methyl-4-hexenoic Acid	0.61	0.66	0.70

* All chromatograms were made using the ascending technique, and the spots were developed with ninhydrin reagent. All the saturated α-amino acids gave purple ninhydrin reactions and 2-amino-3-methyl-4-hexenoic acid gave a yellow ninhydrin reaction.

TABLE 2

Synthesis of Some Aliphatic α-Amino Acids



Amino Acid	Yield, Per Cent	M.P., °C. Decomposition	Empirical Formula	Analysis					
				Calculated Per Cent			Found Per Cent		
				Carbon	Hydrogen	Nitrogen	Carbon	Hydrogen	Nitrogen
2-Amino-3-ethyl-pentanoic Acid*	95	149–150	$C_7H_{15}NO_2 \cdot HCl$	....	....	7.71	....	....	7.71
2-Amino-3-methyl-hexanoic Acid	54	238–240	$C_7H_{15}NO_2$	57.87	10.41	9.65	57.62	10.16	9.63
2-Amino-3-methyl-heptanoic Acid	48	228–230	$C_8H_{17}NO_2$	60.32	10.76	8.80	60.49	10.80	8.89
2-Amino-3-ethyl-hexanoic Acid	53	234–236	$C_8H_{17}NO_2$	60.32	10.76	8.80	60.29	10.87	8.68

* M.p. of the free amino acid 249–250°; reported m.p. 245–249° (18).

g. of product which was subsequently recrystallized from ethanol-water, m.p. 232–234° dec.

Anal. Calcd. for $C_7H_{13}NO_2$: C, 58.69; H, 9.15; N, 9.79. Found: C, 58.23; H, 9.10; N, 10.04.

Results and Discussion

In an effort to determine additional structural specificities of enzymic binding associated with the utilization of valine and/or isoleucine, a number of higher homologs of these natural amino acids were prepared. The first higher homolog of valine may be considered as isoleucine, and the introduction of additional methylene groups into the isoleucine structure was accomplished through the usual acetamidocyanoacetic ester synthesis (1) as indicated by the accompanying equations. In addition to these

$$\text{R-Br} + \text{H-C(CN)(COOEt)-NH-COCH}_3 \xrightarrow[2.\ \text{H}^+]{1.\ \text{NaOEt}} \text{R-CH(NH}_2\text{)-COOH}$$

R = 3-pentyl (I)
3-hexyl (II)
2-pentyl (III)
2-hexyl (IV)

saturated analogs, the 4,5-dehydro derivative of III, 2-amino-3-methyl-4-hexenoic acid (V) was also synthesized in order to determine the effect on biological activity of introducing a carbon-carbon double bond in a higher homolog of isoleucine or valine.[3]

Analogs I through V were tested as antagonists of isoleucine and valine in *L. arabinosus*, *S. fecalis*, *S. lactis*, *L. dextranicum*, and *E. coli*. No significant inhibitory effects were observed in these organisms up to a concentration level of about 400 μg./ml., with the single exception of 2-amino-3-ethylpentanoic acid which

[3] With the exception of 2-amino-3-ethylpentanoic acid (I), all of the other new amino acid analogs herein described contain two asymmetric carbon atoms; thus, for each of the higher homologs, II, III, IV, and V, there are diastereoisomeric structures. In view of the method of synthesis, it may be assumed that both of the anticipated diastereoisomeric forms were produced. Since the analogs possessed only moderate inhibitory activities as subsequently demonstrated, and since isoleucine and alloisoleucine are about equally effective in inhibiting valine utilization under the identical assay condition, exhaustive attempts to separate the analogs into the pure isomeric forms were discontinued.

was toxic to *L. dextranicum* at about 200 μg./ml. The relatively poor solubility of these analogs in the assay medium precluded testing them at higher concentration levels.

During the course of this investigation, specific nutritional mutants of *E. coli* 9723 were made available, and the biological activities of these derivatives were accordingly examined in these organisms. Neither the analogs nor valine possessed any appreciable toxicity for an isoleucineless *E. coli* mutant, grown in a medium containing a minimal amount of isoleucine. However, when these derivatives were examined using a valineless *E. coli* 9723 mutant, containing a minimal quantity of valine, all of the analogs effectively inhibited microbial growth. The toxicities of 2-amino-3-ethylpentanoic, 2-amino-3-methylhexanoic, 2-amino-3-ethylhexanoic, 2-amino-3-methylheptanoic, and 2-amino-3-methyl-4-hexenoic acids are reversed competitively by DL-valine in this valineless mutant with inhibition indices (ratio of analog to valine just necessary for inhibition of growth) of 5, 5, 10, 10, and about 25, respectively. A typical set of data is presented in Table 4 for these competitive reversals using 2-amino-3-methylheptanoic acid as an example.

It was also of interest to compare the relative inhibitory effects of isoleucine and alloisoleucine with the synthetic higher homologs as antagonists of valine utilization in this valineless *E. coli* mutant. Isoleucine and alloisoleucine are both antagonistic to growth of this organism and are competitively reversed by valine

TABLE 4

Reversal of 2-Amino-3-methylheptanoic Acid Toxicity in Valineless Escherichia coli *9723 by* DL-*Valine*[a]

2-Amino-3-methylheptanoic Acid μg./ml.	Supplement: DL-Valine, μg./ml.			
	10	20	50	100
	Galvanometer Readings[b]			
0	72	72	73	73
20	67			
50	66			
100	11	72		
200		10	72	
500			0	71
1000				5

[a] Incubated 15 hours at 37°.
[b] A measure of culture turbidity; distilled water reads 0, an opaque object 100.

with an inhibition index of about 2. At high concentrations (50 μg./ml.), alloisoleucine exerts an inhibition which is not reversed by further increases in valine concentration. These data suggest that an additional inhibitory effect upon a different system occurs at this high concentration of inhibitor. The inhibitory effects of all of these amino acids are indicated in Table 5. On a weight basis, 2-amino-3-ethylpentanoic and 2-amino-3-methylhexanoic acids were only one-half as effective as isoleucine or alloisoleucine in inhibition of growth, and 2-amino-3-ethylhexanoic and 2-amino-3-methylheptanoic acids are only about one-fourth as toxic.

It is of considerable interest that the higher homologs of isoleucine can inhibit the utilization of exogenous valine but not exogenous isoleucine, in the valineless and isoleucineless *E. coli* mutants, respectively. These results indicate that isoleucine in inhibiting the utilization of valine in the valineless mutant does not do so by preventing as assimilation reaction common for both isoleucine and valine as has been previously suggested by some authors (6); otherwise, the analogs should be antagonists of both isoleucine and valine. Apparently, the higher homologs of isoleucine are sterically hindered from binding the enzyme essential for isoleucine utilization but not that for valine utilization. Since

TABLE 5

Relative Toxicities of Various Amino Acids to Growth of a Valineless Escherichia coli *Mutant*[a]

	Supplement: DL-Valine, 10 μg./ml.[b]					
	Inhibitor					
Inhibitor Concentration, μg./ml.	2-Amino-3-ethyl-pentanoic Acid	2-Amino-3-ethyl-hexanoic Acid	2-Amino-3-methyl-hexanoic Acid	2-Amino-2-methyl-heptanoic Acid	DL-Iso-leucine	DL-Alloiso-leucine
	Galvanometer Readings[c]					
0	62	60	61	63	62.5	62
5					59	60
10	52	57	46	61	49	43
20	28	51	43	53	0	2
50	0	47	0	28		
100		11		1		
200		1				

[a] Incubated 16 hours at 37°.
[b] Basal inorganic salts-glucose media described in references (2, 9) was used.
[c] A measure of culture turbidity; distilled water reads 0, an opaque object 100.

isoleucine and alloisoleucine are about equally effective in inhibiting valine utilization, either methyl group of valine may be extended without appreciably altering ability to bind the enzyme. Ability to inhibit endogenous synthesis and utilization of valine in the wild type *E. coli* is more restricted to specific structures, and the higher homologs of valine are ineffective as inhibitors under these conditions.

Summary

Several higher homologs of valine and isoleucine (2-amino-3-ethylpentanoic, 2-amino-3-methylhexanoic, 2-amino-3-ethylhexanoic, 2-amino-3-methylheptanoic, and 2-amino-3-methyl-4-hexenoic acids) were prepared through the condensation of the appropriate alkyl bromide and ethyl acetamidocyanoacetate, followed by hydrolysis of the intermediate condensation product. A microbiological study was then made concerning the specificity of the terminal grouping of these α-amino acids to compete with the natural substrates for essential enzymes. These compounds did not have any appreciable toxicity for a number of organisms including an isoleucineless mutant of *Escherichia coli* 9723; however, they were inhibitory to growth of a valineless *E. coli* mutant, and were competitively reversed by supplements of valine.

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MORPHOLOGICAL AND CYTOLOGICAL STUDY OF HUMAN AMNION CELLS IN THE COURSE OF RECOVERY FROM RADIATION INJURY[a]

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An increase in the size of cells, or of their nuclei, following irradiation has been described for cells in tissue culture, cancer cells, and microorganisms. Paterson (1942) seems to be the first who has reported on giant cell formation in culture material following x- and gamma irradiations. She observed that some elements became enlarged in the second subculture of chick choroid and sclera *in vitro* under irradiation at low dosage. More recently, Puck and Marcus (1956) have shown that an increase of X-ray dosage has induced a decrease in the number of HeLa cells, with an increase in the number of giant cells. Pomerat *et al.* (1957*a*) have reported that giant cell formation following irradiation is typical in established *in vitro* strains, but not in primary cultures. Following these pioneer workers, many investigators have dealt with giant cell formation as a result of radiation injury (4,6,13,14,20–22, and others).

Pomerat and his co-workers have postulated that giant cell formation may take place in association with an increase in number of the chromosomes in cells that were derived from the subcultures of primary outgrowth. The mechanism of giant cell formation has, however, still remained in question.

In most cases, the observations of giant cells have been performed some time after irradiation. In the present study, the morphological and cytological changes in human amnion strain

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cells were checked quantitatively during the first five days following gamma irradiation from a cobalt^{60} source. In addition, the change in modal value of chromosome numbers was studied during this time.

Materials and Methods

A well established human amnion strain (Fernandes) (9,14) was cultivated in T-60 flasks. When the cell growth reached maximum, a rubber policeman was employed to scrape the cells from the glass surface. Cells were dispersed with a 30 ml. syringe fitted with a #15 needle, and transferred to a 50 ml. flask in order to count cell population with a hemocytometer. A standard number of 10,000 cells was seeded initially in each Rose chamber (19) two days prior to irradiation. Eagle's nutrient medium was used with 10 per cent horse serum.

Cultures were irradiated in the high-level cobalt^{60} facility at the Radiological Laboratory of The University of Texas and the United States Air Force in Austin, Texas. Gamma irradiation was delivered to the field of irradiation at a rate of 2000r per minute for 1000r. The nutrient medium was removed immediately before irradiation, and fresh medium was supplied immediately after the treatment of the cells.

Irradiated cells in Rose chambers, together with unirradiated controls, were fixed with methyl alcohol and stained with a combination of dilute Giemsa and May-Grünwald according to Jacobson's method, daily for five days following irradiation. Details of the methods used for cell counting will be described in the following section.

Results

Cell population:

After a lag of one day, irradiated cells began to detach from the glass surface of each chamber. For the quantitative study of stained preparations, total population counts (inclusive of both the non-giant and giant cells) were made in terms of the number of cells in a standard area. This area was defined by a reticule, being made up of a path 0.5 mm. wide across the diameter of the chamber, or a total area of 14 mm.^{2} when determined with the use of a 10x ocular and a 10x objective. Generally the cells were not evenly distributed throughout the chamber; therefore it was necessary to determine the average of counts across four different axes of each chamber to insure an accurate sampling. These average values were used to calculate the number of surviving cells in relation to a standard of 1000 cells in the unirradiated control cultures. The cell population curve is shown in Figure 1. The number of cells in the control cultures increased

until a plateau was reached at four days. After the fourth day the number of cells began to decrease, perhaps due to an overgrowth of cells in the chambers. In contrast, irradiated cultures showed a progressive decrease in cell numbers during the observation period.

Rose chambers were used for a cinematographic study of irradiated cells during the first three days following 1000r radiation. Most cells became rounded for the moment, and finally broken

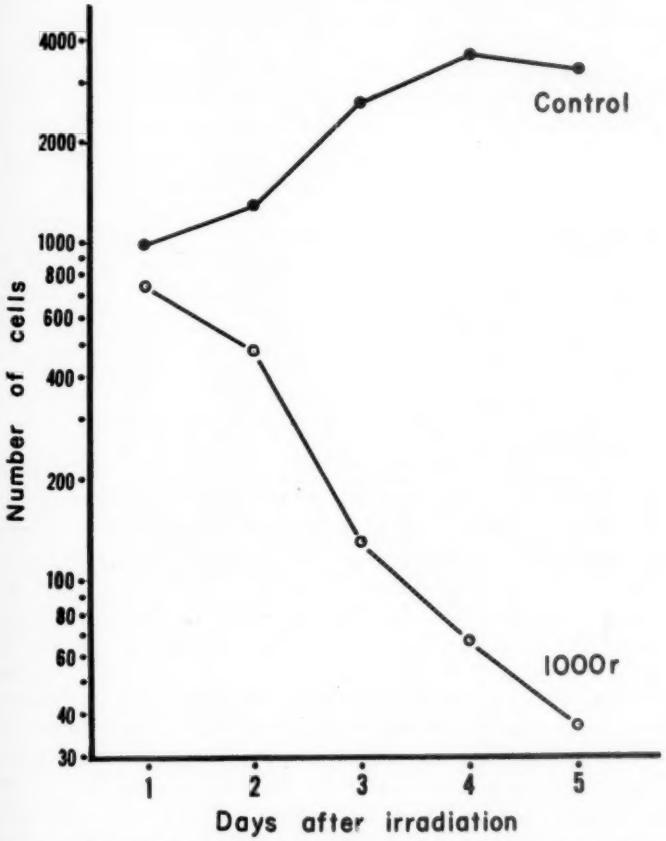


FIG. 1. Cell population curve in irradiated cultures during five days post-irradiation (1000r), together with control.

down, becoming free from the glass surface of the chamber. No other picture of degeneration of irradiated cells has been observed, at least in this record.

Mitotic index:

The mitotic index for both control and irradiated cultures in each group was determined, based on the observations of a total of over 2000 cells. As seen in Table 1, the mitotic index in non-irradiated cultures indicated a maximum value of 5.6% on the

TABLE 1

The mitotic index of irradiated human amnion cells during 5 days following 1000r from a cobalt^{60} source, together with unirradiated control

	Prophase	Metaphase	Anaphase	Telophase	Total Cells	Mitotic Index (%)
			CONTROL			
1st day	8	88	8	15	2127	5.6
2nd day	6	79	7	15	2020	5.3
3rd day	10	58	8	12	2079	4.2
4th day	8	55	5	12	2014	4.0
5th day	6	49	7	10	2070	3.5
			TREATED			
1st day	1	14	1		2017	0.8
2nd day	3	177	2	3	2030	9.1
3rd day	5	315	3	3	2058	15.7
4th day	1	100			2025	5.0
5th day	1	46	1	1	2132	2.3

first day (three days after setting up of cultures); then it showed a gradual decrease. In the irradiated cultures, the mitotic activity of the cells was remarkably inhibited on the first day (0.8%), but it showed a sudden increase on the second day (9.1%), and reached maximum on the third day (15.7%). Then the mitotic index fell rapidly (2.3%), along with a decrease of cell population.

Though the mitotic index on the second and third day following irradiation showed a remarkable increase, it may not indicate a real value of the mitotic activity of irradiated cells; the high values may result from the accumulation of the cells at metaphasic stage (Table 1). The number of cells at prophase, anaphase, and telophase in irradiated cultures was much lower than that on non-irradiated control through five days of observa-

tion. The analysis of 16 mm. film records for the control revealed that 36 cells which entered into prophase underwent regular cell division. On the other hand, in the irradiated cultures only three out of 34 cells which reached metaphase divided regularly.

A T-60 flask of a human amnion strain that was irradiated at a dosage of 1000r was treated with hypotonic solution (sodium citrate 1.12% in distilled water) for 10 minutes on the third day post-irradiation, and fixed with freshly prepared Carnoy's fluid. Then the cells were stained slightly with acetic orcein, squashed, and the chromosomes were observed under a phase microscope. In most metaphasic figures the chromosomes showed various types of abnormalities such as fragmentation, stickiness, and clumping (Figures 2 and 3). These severe chromosomal aberrations might inhibit migration of chromosomes to the poles at anaphase, so that metaphasic cells underwent degeneration in this condition without advancing further.

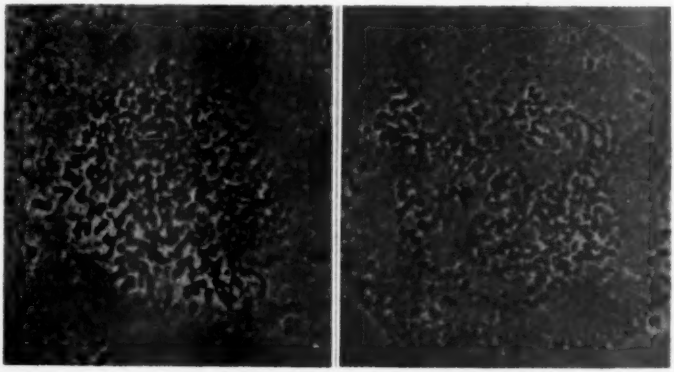


FIG. 2–3. Chromosome anomalies in human amnion cells induced by 1000r from a cobalt^{60} source.

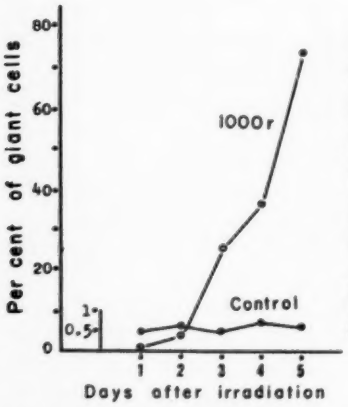
Giant cells:

Radiation-induced giant cells were arbitrarily defined as being elements which had at least three times the area of the cytoplasm of the non-giant cell. These giant cells were usually characterized by bizarre multilobed nucleoli and nuclear membranes showing wrinkled surfaces and marked indentations. The percentage of giant cells was calculated by counting the number of giants in a minimum of 2000 cells for each experimental condition. As seen

in Figure 4, control cultures showed a constant value of giant cells for five days after irradiation (0.5–0.7%), while the incidence of giant cells on the second day was already higher in the irradiated cultures than in the controls. Then it increased remarkably with the progress of time. This increase in incidence seems to be due in part to a decrease in the total cell population, and therefore it seems necessary to examine the average number of surviving giant and non-giant cells in comparison with the standard number (1000) of cells in control cultures. As seen in Figure 5, giant cell formation increased at a higher rate in irradiated cultures than in the controls, and reached a maximum on the third day after irradiation.

Multinucleate cells:

The number of multinucleate cells, consisting of both giant and non-giant elements, significantly increased in irradiated cultures, as compared with that in control cultures (Figure 6). The rate of the increase of multinucleate giant cells was linear from the second day (3.6%) to the fifth day (35.3%). In contrast,



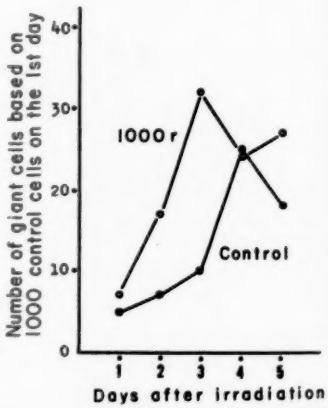


FIG. 4. Graph showing the incidence of giant cell formation after 1000r, together with that of control. Giant cells increase lineally in experimental cultures, while control shows a constant percentage of giant cell formation during five days following irradiation.

FIG. 5. Graph showing the number of giant cells in each of five days after 1000r. Giant cell formation increases at greater rate in irradiated cultures than in the control during the first three days after irradiation.

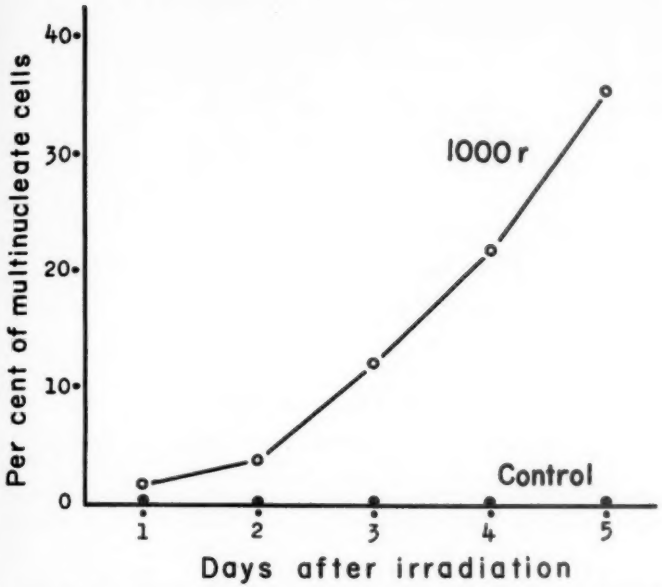


FIG. 6. Irradiation (1000r) significantly increases the number of multinucleate cells without lag, as compared to control.

there was no change in the incidence of multinucleate cells in the control series.

In order to make clear the relationship between the formation of multinucleate cells and days after irradiation, cells were classified into four groups: multinucleate giants, mononucleate giants, multinucleate non-giants, and mononucleate non-giants. The numbers in each group were recalculated on the basis of the standard number (1000) of cells in the control population. The appearance of multinucleate giant cells in irradiated cultures reached a plateau between the second and fifth day, while in control cultures no change in number was observed (Figure 7). In control cultures, an increase in number of mononucleate giant cells was seen with a maximum on the fourth day. Irradiated cultures, however, showed the maximum number of mononucleate giant cells on the third day (Figure 8). The mononucleate non-giants underwent rapid damage by irradiation. The multinucleate non-giants were found in large number during the first

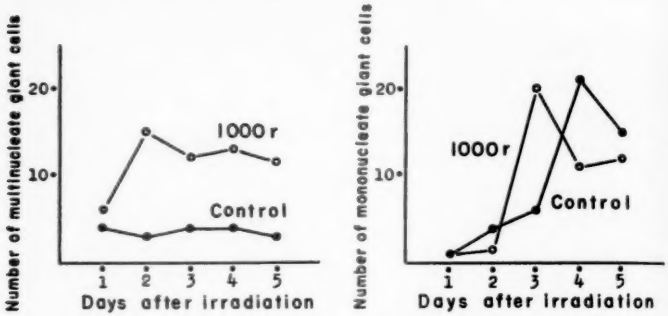
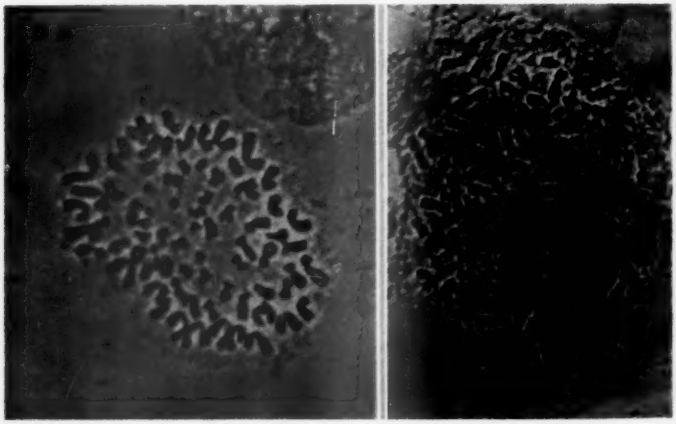


FIG. 7. The appearance of multinucleate giant cells reaches a plateau between the second and fifth days following 1000r.

FIG. 8. Irradiation (1000r) appears to increase the rate of formation of multinucleate giant cells, with a peak reached on the 3rd day.



FIGS. 9–10. Photomicrographs of metaphase chromosomes in human amnion strain cells in control and irradiated cells (1000r).

FIG. 9. A control cell having 75 chromosomes in 84th passage.

FIG. 10. A cell having 73 chromosomes in the 35th subculture of subline R-1.

three or four days in irradiated cultures (3–6%), while in the control series their rate was 0–2%.

Change in modal value of chromosomes:

On the fifth day following 1000r irradiation, approximately

70 per cent of cells were giant elements. On the seventh day, however, some colonies of non-giant cells were found in an irradiated flask. Therefore the first subculture was made, using a rubber policeman and T-60 flasks. A flask was sacrificed for the chromosome spreading. The method used for the spreading was the same as that reported by Nakanishi *et al.* (1959). Chromosomes were counted from 50 reliable metaphasic plates. The results are given in Figure 11. In the control (84th passage), the most frequent chromosome number was 75 (31 out of 50 cells), with a range of from 69 to 79 (Figure 9). On the other hand, the experimental culture showed the chromosome numbers distributed over a wide range from 60 to 135, with small peaks at 71, 72, 73, 74, and 75. In addition to this anomaly, dicentric chromosomes appeared in 10 per cent of the metaphase plates examined.

After the third subculture, a new modal chromosome number appeared at 73 (21 out of 50 cells examined). The dicentric chromosomes no longer appeared in this passage. In the sixth subculture, the modal value of chromosomes was apparently 73, and the frequency of the modal value was higher than the third subculture (26 out of 50 cells). This modal value was also observed in the 18th and 35th subcultures (Figure 10). The modal value of a control culture as passage 92 was still 75 (19 out of 30 cells). In the light of the above findings, it seems apparent that a new subline had been established. Further experiments using this new subline are being reported by the authors (8).

Discussion

The effects of ionizing radiation on living cells have been demonstrated by various types of alterations in their morphology and behavior. Pomerat *et al.* (1957*a*) used phase contrast time-lapse cinematography techniques to analyze the changes in cultures of living human strains one to five days following irradiation. According to these investigators, cell enlargement and teloreduplication, including a second division, have regularly taken place in treated materials. Furthermore, the multinucleation which arose from cells with single or multiple nuclei, and produced one or two daughter cells forming numerous micronuclei, was found for all three strains used for their observations *in vitro*. A similar situation has been found to occur in the present study. The reduction of cell population, disturbance of anaphasic

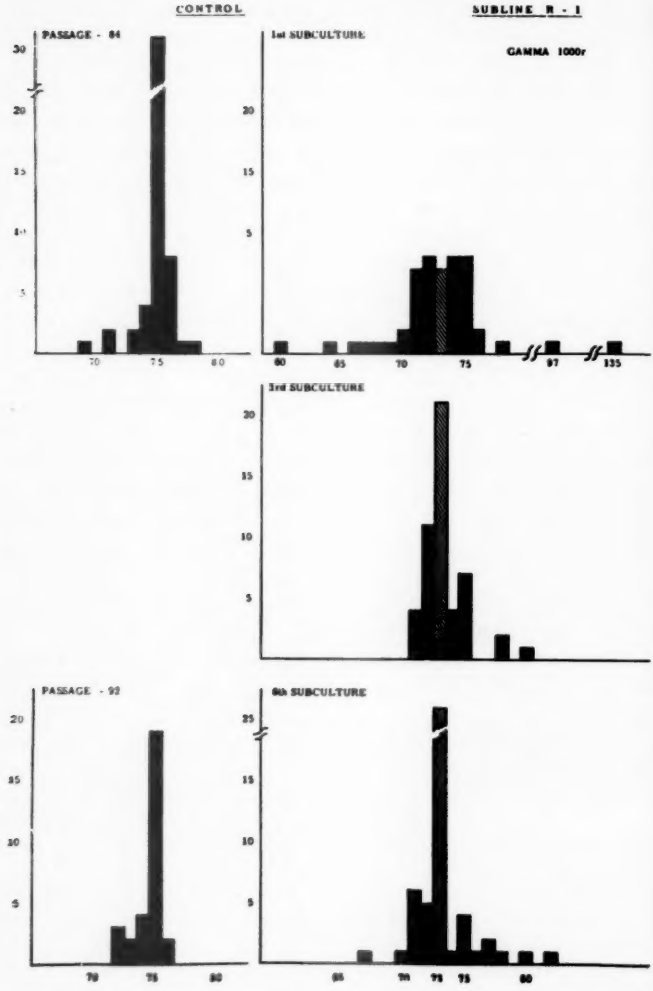


FIG. 11. Distribution of chromosome numbers in cells in subline R-1 treated with 1000r from a cobalt^{60} source, and in the untreated cultures.

movement of chromosomes to the poles, the numerical increase of giant cells, and the increase of multinucleate cells occurred rather commonly.

One of the important causes for the decrease in cell population appears to be the death of cells resulting from the damage of chromosomes. Puck (1958) has described the relationship between the death of the cells and chromosome anomalies resulting from X-radiation on human euploid cells *in vitro*. He concluded that the primary cause leading to the inhibition of mitosis was usually the chromosome damage. As the squashed preparation for chromosome analysis showed on the third day following 1000r irradiation that the chromosomes were affected grossly, there is no doubt that the chromosomal anomalies were so severe that anaphasic separation of chromosomes was virtually impossible, thus leading to cell damage. This assumption may be supported by the fact that there are few anaphase and telophase figures in irradiated cultures.

Giant cells under study increased lineally from the second day to the fifth day after irradiation. However, the number of those elements based on the cell population curve reached a maximum on the third day. This indicated that the decrease of irradiated cell population was due in part to the degeneration of the giant cells after the third day following irradiation, through the giant cell formation continued at a slow rate. The degeneration of giant cells in the early days post-irradiation was remarkable in mononucleate giant elements (Figure 8). Tolmach and Marcus (1960) have observed that in irradiated HeLa S3, cell enlargement continued exponentially for at least six days after irradiation, and the rate of enlargement decreased, reaching zero about 12 days post-irradiation. Such cell populations may be maintained for several weeks (15,22).

Many reports have been published concerning giant cell formation following ionizing radiation, but the mechanism of giant genesis has remained unexplained. Pomerat *et al.* (1957*b*) showed that gamma irradiation from a cobalt^{60} source served to produce giant cells within 12 days in a human strain, while primary explants that were cultivated for about one month showed no giant cell formation. On this basis they postulated that the above results might be connected with an increase in number of the chromosomes in cell strains. It seems very probable that the appearance of giant cells in strains may be due to chromosome imbalance.

In general, neoplasms characterized by a chromosome poly-

ploidy are more refractory to X-rays than those of a diploid condition. Hauschka *et al.* (1959) expressed the view that stem cells in neoplasms might be monosomic as a result of deletions and translocation of chromosomes, and such neoplasms having haploid karyotypes might be more vulnerable to ionizing radiation than the cells which are diploid. According to Bacq and Alexander (1955), the radiation sensitivity of yeast, higher plants, and insects is correlated with the number of sets of chromosomes. In other words, haploid species are more radiosensitive than diploid ones.

If the cells are prevented from dividing, but continue to grow at a normal rate, the size of the cells may be increased instead of their number (7). Tolmach and Marcus (1960) concluded that the giant cell formation might be attributed to ultimate failure of the division process. It is evident from Table 1 that in these studies the mitotic activity of irradiated cells was inhibited for five days following 1000r. From the morphological aspects, therefore, giant cell formation might be attributed to the inihbition of mitotic activity, in addition to the teloreduplication (which is defined as a simple fusion of daughter cells during or after cytokinesis), and to the formation of multinucleate cells without cytokinesis.

Casperson *et al.* (1958) reported that radiation of ascites tumor cells with 1250r of X-rays resulted in an increase in protein and nucleotide content. Sheek *et al.* (1960) used radio-isotopes in order to follow the course of DNA and protein syntheses during the formation and growth of giant cells. They showed that thymidine-2-C^{14} uptake into cellular deoxyribonucleic acid continued at a normal rate for 10 days after irradiation and then declined, while glutamic acid-C^{14} uptake was rapid, after six days reaching a greater rate than that of normal cells. It is logical to conclude that, while the mitotic mechanism is inhibited by radiation, DNA and protein syntheses may be normal or even accelerated.

After the degeneration of dividing cells, some survivors could grow into new subclones. Kohn and Fogh (1959) observed that there was no significant difference in growth rate and cell size between cells in subcultures derived from 1000r irradiated human amnion cells and those from their original source. However, the present results indicate that the chromosome numbers shift in

irradiated cells after the recovery from radiation injury. Puck (1959) has reported that four out of five new stocks derived from radiated single cells of HeLa S3 had mutated from the original one, exhibiting morphological and nutritional differences which persisted after a long growth period. Chu and Giles (1958) have reported that some mutated sublines possessed chromosome numbers different from those of the parent lines. Rhynas and Newcombe (1960) have observed that all radio-resistant lines of L-strain that received accumulated 4000r and 5000r showed lower modal chromosome numbers than the controls.

In the present obeservations the modal value of chromosomes in the newly established subline have been maintained constantly at 73, in contrast to 75 in the parent line. This gradual shift in the modal value of chromosomes toward lower numbers with successive passages of cultures is suggestive of selective adaptation. More details of this problem are discussed in a paper published by the authors in 1961 (8).

Summary

A well established human amnion strain which was cultivated in Rose chambers was irradiated with 1000r from a cobalt^{60} source. The materials thus treated were stained with Jacobson's method daily for five successive days following irradiation. The changes in cell population and mitotic index and the incidence of giant and multinucleate cells were examined. A population of this strain which had a modal chromosome number of 75 showed severe injury after irradiation. The cell cultures grown from the recovery of radiated cell cultures were characterized by a modal chromosome number of 73. The mechanism of giant cell formation and the changes in the modal chromosome numbers have been discussed in reference to reported evidence.

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CHANGES IN DEOXYRIBONUCLEIC ACID DURING RENAL COMPENSATORY HYPERTROPHY IN THE RAT

KAZUO OGAWA[a,b]

In 1948, Boivin *et al.* and Vendrely and Vendrely suggested that the content of deoxyribonucleic acid (DNA) per nucleus in the somatic cells of different tissues of the same species is constant and that there is a direct relation between the amount of DNA and the chromosome polyploidy. Since that time, much work has been carried out in support of this concept (Leslie, 1955), and, despite a limited evidence against it, it is universally accepted as a working hypothesis. Of course, the concept of constancy of DNA per nucleus is valid in normal cells and under physiological conditions. In the regenerating rat liver, for instance, an increased amount of DNA per nucleus was obtained by Thomson *et al.* (1953). Tsuboi *et al.* (1954), using chemical methods of estimation, found an increased amount of DNA per nucleus. Fautrez *et al.* (1955), using Lison's histophotometer, showed a similar phenomenon in the compensatory hypertrophy of the kidney, 7 days after unilateral nephrectomy. Mangiore (1953), however, observed a gradual decrease of ribonucleic acid (RNA) and DNA in cells of the tubules under similar conditions.

In the present paper we attempted to solve this discrepancy by estimating chemically the amount of DNA per nucleus in the kidney during compensatory hypertrophy at different days following unilateral nephrectomy.

Materials and Methods

Holtzman strain male albino rats, weighing 153 to 176 gm., were used. They were nephrectomized on one side. On the 2nd, 3rd, 5th, 7th, 10th, 20th, 30th, 40th, and 50th postoperative days respectively, rats were sacrificed, the remaining kidneys immediately removed and immersed in ice-cold saline solution. They were then decapsulated, gently dried on filter paper and weighed. Whole kidneys were homogenized in a Virtis homogenizer with ice-cold 0.04 M citric acid solution; the final concentration was 5 per cent. One drop of caprylic alcohol ethanol mixture (1:4 v/v) was added to reduce

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occasional foaming. The amount of DNA per nucleus was estimated by the method described in detail by Nowinski and Yushok (1953).

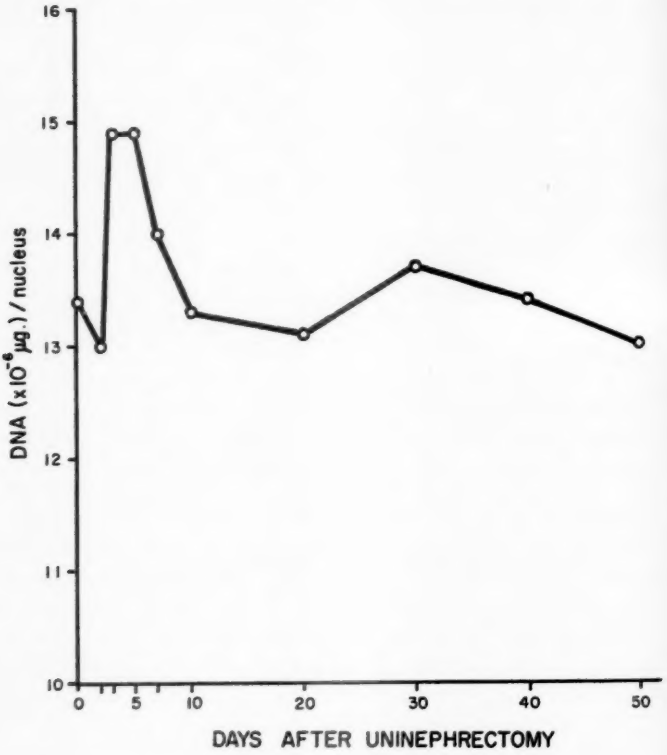

FIG. 1. DNA content per nucleus at progressive stages of compensatory hypertrophy of the kidney.

Results

Figure 1 represents graphically the results obtained: the DNA content per nucleus in the normal rat kidney was 13.4×10^{-6} µg (Fig. 1, Table 1). On the second day following nephrectomy, the DNA content per nucleus in the remaining kidney was 13.0×10^{-6} µg. On the 3rd day, the quantity of DNA per nucleus increased to 14.9×10^{-6} µg. (The t value for the difference between

TABLE 1

The DNA Content per Nucleus During the Compensatory Hypertrophic Kidney of the Albino Rat

Days after Uninephrectomy	Number of Rats	DNA ($\times 10^{-6}$ μg.)/Nucleus
Normal	16	13.4 ± 0.76*
2	6	13.0 ± 1.33
3	6	14.9 ± 1.27
5	6	14.9 ± 0.76
7	2	14.0 ± 0.28
10	6	13.3 ± 0.87
20	6	13.1 ± 0.52
30	6	13.7 ± 0.90
40	6	13.4 ± 0.22
50	6	13.0 ± 0.89

* Mean ± standard deviation.

the values for the 2nd and the 3rd days was 2.7, the difference not being significant, but the t value for the difference between the values for the normal and 3rd day was highly significant: 4.4. The DNA content per nucleus on the 5th day was 14.9×10^{-6} μg. (t value for the difference between the data of the 2nd and 5th days was significant: 3.1). On the 7th day, the DNA still remained at a higher level: 14.0×10^{-6} μg. (t value for the difference between 5th and 7th day was 1.5, not significant).

The DNA content per nucleus on the 10th day after the operation was within the normal range: 13.3×10^{-6} μg. However, the difference between the values for 5th day and 10th days was still significant. On and after the 20th postoperative day, values for the DNA content per nucleus dropped to essentially normal: 13.1×10^{-6} μg., 13.7×10^{-6} μg., 13.4×10^{-6} μg., and 13.0×10^{-6} μg. on the 20th, 30th, 40th and 50th postoperative days, respectively.

These findings show that the DNA content per nucleus in the hypertrophic kidney increases within 3 days after unilateral nephrectomy and then returns within 10 days to normal values.

Discussion

In the present investigation, the DNA content per nucleus in the normal male rat kidney (Holtzman strain) was found to be 13.4×10^{-6} μg. Kurnick (1951), however, gave the average DNA

content of the rat kidney nucleus to be 9.3×10^{-6} μg., in rats weighing 80 to 219 gm. Harrison (1951) and Leuchtenberger *et al.* (1951) obtained values of 5.7×10^{-6} μg. and 5.5×10^{-6} μg. respectively. Harrison used the cortices of kidneys from rats weighing approximately 200 gm. Thomson *et al.* (1953) found the average DNA content per nucleus in the kidneys of normal adult rats (195 to 250 gm.) to be 6.64×10^{-6} μg. and in those of normal young rats (35 to 90 gm.) 7.02×10^{-6} μg. The value obtained in the present investigation was almost twice as high as those obtained by these authors. A similar discrepancy was found by Nowinski and Yushok (1954) on chick embryo nuclei. This difference might be due to the variations in method of determination and in strains of rats used, or to possible errors in the method of counting nuclei. In the present study such differences do not affect the findings, which are based on a comparison between the contents of DNA in nuclei of normal kidneys and that in compensatory hypertrophy. Thus, the relative values: normal-hypertrophy are of importance, irrespective of whether the absolute values are in agreement with other authors.

In the regenerating mouse liver after partial hepatectomy, Tsuboi *et al.* (1954) found an increased amount of DNA per nucleus, which returned to normal on the 8th postoperative day. Further, Thomson *et al.*, in the previously mentioned paper, showed that in the regenerating rat liver after partial hepatectomy, the DNA content per nucleus was 10–50% above the mean value during the first 4 days, as compared to normal rats of the same sex, strain and body weight.

According to Bader (1953), tumor tissue also contains an increased amount of DNA per nucleus as compared to normal tissue, and this increase was considered to be connected with changes in chromosome content, such as polyploidy, and not attributed to a disturbance in the synthesis of nucleic acid. Leuchtenberger *et al.* (1954), using the microspectrophotometric method, could show, indeed, that there is a wide variation in the DNA content per nucleus from cell to cell in tumors and they also regard it as a result in abnormal mitotic activity. Similarly, Tsuboi *et al.* also suggested an increased incidence of polyploid nuclei in the regenerating mouse liver which might be responsible for the increase of DNA per nucleus during the process of regeneration. Thomson *et al.* (1953), however, interpreted this

increase as being due primarily to the higher rate of mitosis during regeneration.

Since the reports of Caspersson in 1939 and Ris in 1947 that in the mitotic cycle the doubling of DNA of the nucleus begins at prophase and is completed at metaphase, a great deal of work has been devoted to a study of the time of doubling of the nuclear DNA content. It seems to have been established that DNA is synthesized during the interphase before the visible morphological onset of prophase in the nucleus (for literature see Sisken, 1957). Therefore, in tissues undergoing a high rate of mitosis, considerable synthesis of new DNA must occur, the DNA content per nucleus thus increasing. In the case of unilateral nephrectomy, the remaining kidney shows an immediate response, and mitotic activity increases considerably within 2 days and stays so up to approximately the 10th postoperative day (Rollanson, 1949; Sulkin, 1949; Ogawa and Sinclair, 1958). This also is the case with the regenerating liver (Wilson *et al.* 1953). Furthermore, Barnum and others (1957), using P^{32}, showed in the regenerating rat liver after partial hepatectomy, that the DNA turnover rate increased within 36 hours after the operation, and returned to normal within 72 hours. According to Barton (1954) and Taylor *et al.* (1957), once DNA has become part of a chromosome it is biochemically stable even during chromosome duplication and cell division. Thus, the increased DNA turnover rate noted by Barnum and others implies that the synthesis of new DNA is occurring quite rapidly in the regenerating liver. We have the right to assume that an identical or a similar mechanism may be involved in compensatory hypertrophy of the kidney.

No work has been reported on the possible occurrence of polyploid nuclei in the compensatory hypertrophic kidney after unilateral nephrectomy. However, in the regenerating rat liver, (Makino and Tanaka, 1953) the ratio of pre-existing polyploid nuclei to the diploid nuclei increases about 100 per cent within 24 hours after operation and then decreases to normal within 96 hours. Beams and King (1942) also found that the regenerating liver contained a great number of polyploid cells. Though normal liver contains many polyploid cells, the percentage of polyploid cells still appears to increase transiently during its regeneration Heimes *et al.* 1957). The observed increase in DNA content per

nucleus in the compensatory hypertrophic kidney might also be due partly to the occurrence of polyploid cells, even if there exists no polyploid nucleus in the normal kidney. According to Heimes *et al.*, the average DNA content of nuclei following partial hepatectomy remains slightly above normal after the initial transient increase. This initial increase is considered to be due primarily to an increased number of mitoses; however, the latter persistent slight increase above normal is due to an altered ratio of polyploid to diploid nuclei in regenerating liver. The return to a normal value following the initial increase which we observed in the case of compensatory hypertrophy of the kidney implies that the occurrence of polyploidy after unilateral nephrectomy is not very likely.

Fajers (1957) found that in the kidney undergoing compensatory hypertrophy the mean nuclear volume of the proximal convoluted tubule increased in 24 to 48 hours and subsequently showed a return to normal size by the 10th postoperative day; this he considered to be a sign of increased cellular activity. The nuclear volume seems not to be related to the amount of DNA per nucleus (Swift, 1950, 1950a), but is rather directly connected with protein synthesis, as shown by a number of investigators (Schrader and Leuchtenberger, 1950; Alfert *et al.* 1955 and 1955a), and with the synthesis of ribonucleic acid (Sisken, 1957).

Summary

1. The deoxyribonucleic acid (DNA) content per nucleus was measured in the rat kidney undergoing compensatory hypertrophy following unilateral nephrectomy.

2. In compensatory hypertrophy of the kidney, the DNA content per nucleus increases within 3 days after the operation and returns to normal within 10 days; the probable causes of this phenomenon, such as increase in mitotic rate, polyploidy, etc., are discussed and compared to the DNA changes in regenerating liver.

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DEOXYRIBONUCLEOSIDE METABOLISM BY RAT ERYTHROCYTE EXTRACTS*

D. A. Rappoport, P. T. Chen,† and R. R. Fritz

While investigating purine ribonucleoside metabolism by rat erythrocyte extracts (10, 12) it was noted that purine deoxyribonucleosides were also metabolized by these extracts. Lowy, *et al.* (7) reported that deoxyribonucleosides were metabolized *in vitro* by human erythrocytes to form lactic acid; but they did not attempt a detailed analysis of the intermediates formed in these reactions. In order to clarify the mechanism of purine deoxyriboside dismutation, a study was made of the products formed in the metabolism of deoxyguanosine, deoxyinosine, and deoxyadenosine by rat erythrocyte extracts. This report summaries the results of studies on the nature of intermediate compounds formed from the above substrates.

Experimental

Erythrocytes were separated from fresh cold rat blood by centrifugation and washed four times with cold isotonic KCl. The white cell layer was removed by means of suction during the above washing procedure. The erythrocytes were then lysed in two volumes of cold 0.1 M nicotinamide or distilled water, and dialyzed against 0.0125 M Tris buffer (pH 7.3) overnight at 5–7° C. The dialyzed hemolysate was then centrifuged at 25,000 × g for 30 minutes at 5° C. and the clear supernate was used for studies on deoxyribonucleoside metabolism.

Incubation mixtures consisted of 1.0 ml. of dialyzed erythrocyte extract, varying concentrations of deoxyguanosine, deoxyinosine or deoxyadenosine, 10 μmoles KH_2PO_4-P^{32} (10–15 μC of P^{32}), 1.0 μmole $MgCl_2$, in 0.2 M Tris buffer (pH 7.0) to a total volume of 2.0 ml. At varying time intervals samples of the incubation mixture were deproteinized with cold trichloroacetic acid (TCA) to a final concentration of 10 percent. The supernatant solution was then separated from the protein by centrifugation.

Samples of the supernatant solution were analyzed for deoxyribose by the Stumpf (13) cysteine procedure, or for ribose by the orcinol method (8); for total phosphate esters by a modification of the Martin and Doty method (6)

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and for lactic acid by the procedure described by Barker and Summerson (2). Purines and purines nucleosides were identified by their absorption spectra determined with a Beckman DU spectrophotometer and by means of paper chromatography (3). Deoxyribose 5-phosphate was prepared from deoxyguanylic acid as described by Lampen (5).

Portions of the clear supernatant solution were extracted twice with two volumes of ether to remove TCA. The aqueous phase was then treated with an equal volume of Dowex 50-H^+ resin (freed from excess water by suction) to remove cations. Aliquots of the treated solution were evaporated to dryness in a vacuum desiccator over anhydrous $CaCl_2$. The residue was then dissolved in 1/10 of the original volume of distilled water and aliquots were used for one and two dimensional paper chromatography according to the procedure of Rappoport and Chen (11). The individual phosphate esters were identified by chromatography with the corresponding known ester and by specific color reactions such as that used for deoxyribose phosphate (1).

To obtain data on the rate of individual phosphate ester formation, the esters formed from deoxyguanosine were separated by one dimensional chromatography, their positions were located by means of a radioautogram, and the area for each ester was cut out, mounted on cardboard planchets, and the P^{32}-content determined with a Geiger-Müeller counter (11).

Results

Figure 1 illustrates the rate of metabolism of the deoxypentose moiety from deoxyguanosine and deoxyinosine ,and the simultaneous formation of phosphate esters from each of these substrates. Both substrates are metabolized at about the same maximal rate for 120 minutes and subsequently at a reduced rate for an additional interval of 120 minutes. The rate of phosphate ester formation is maximal for 60 minutes, although the rate of formation from deoxyinosine is slightly lower than from deoxyguanosine. The quantities of lactic acid formed were 0.55 μmole per μmole of substrate utilized.

Table 1 illustrates the amount of deoxypentose moiety utilized from deoxyguanosine, deoxyinosine, deoxyadenosine and deoxy-

TABLE 1

Utilization of Deoxyribose from Nucleosides and from Deoxyribose Phosphate

	Percent
Deoxyguanosine	68
Deoxyinosine	56
Deoxyadenosine	46
Deoxyribose 5-phosphate	70

Conditions as described in the experimental section using 2.0 μmoles of each substrate. Incubated for 2 hours.

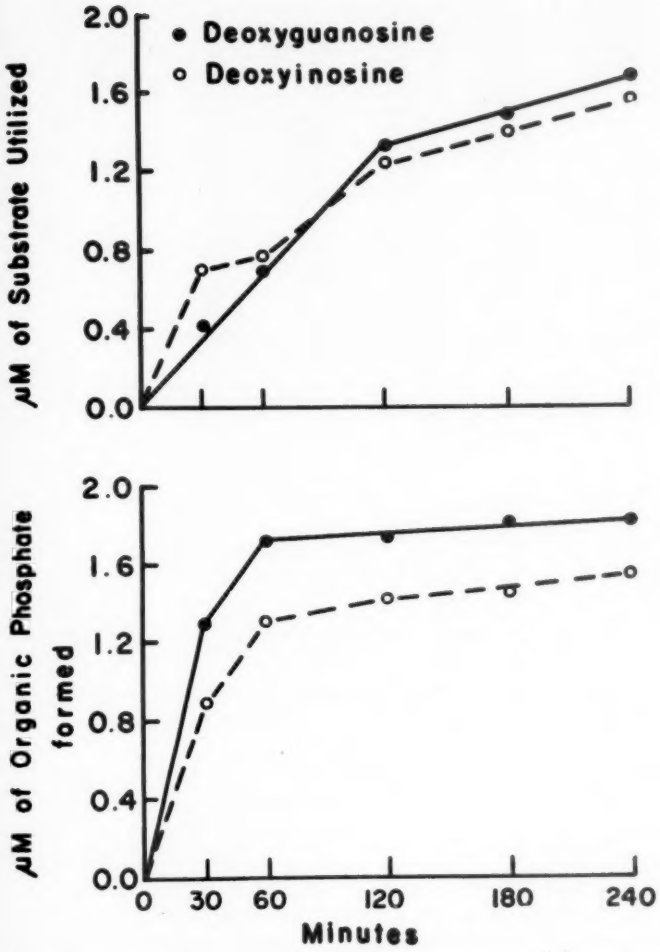


FIG. 1. Rate of deoxyguanosine and deoxyinosine metabolism by rat erythrocyte extracts and the rate of organic phosphate ester formation. Conditions of incubation same as described using 2.0 μmoles of each substrate in each beaker.

ribose 5-phosphate. Deoxyribose 5-phosphate and deoxyguanosine are more extensively utilized than deoxyinosine or deoxyadenosine.

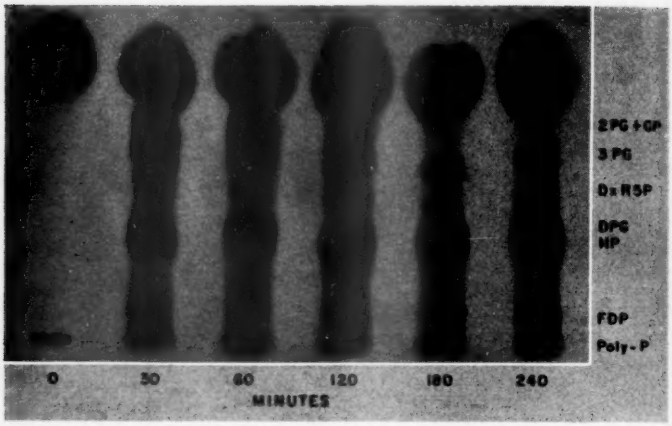


FIG. 2. Radioautogram of a single dimension paper chromatogram of phosphate esters formed from deoxyguanosine incubations with rat erythrocyte extracts.

Figure 2 is a photograph of a radioautogram made from a one dimensional paper chromatogram of the phosphate esters formed from deoxyguanosine incubations during a time study. Similar results were obtained with deoxyinosine and deoxyadenosine. Figure 3 shows a radioautogram of a two dimensional paper chromatogram of the esters formed from deoxyguanosine after 2 hours incubation. Figure 4 illustrates the rate of formation of hexose phosphates plus diphosphoglycerate (HP + DPG), fructose diphosphate (FDP), 3-phosphoglycerate (3PG), deoxyribose 5-phosphate (DxR5P), and 2-phosphoglycerate plus glycerol phosphate (2PG + GP). The maximal formation of 3PG occurred in 30 minutes, 2PG + GP in 1 hour, and the rest of the phosphate esters reach maximal concentration in 2 hours.

Lactic acid formed from the metabolism of pentose moieties from deoxyribonucleosides represents only a portion of the pentose molecule utilized, hence acetaldehyde was considered as an additional product from this dismutation. Using $NaHSO_3$ in a center-well as a trapping agent for acetaldehyde (9), incubations were tested for acetaldehyde formation from deoxyguanosine and deoxyribose 5-phosphate using stoppered containers. Table 2 illustrates these results. These values represent an approximate

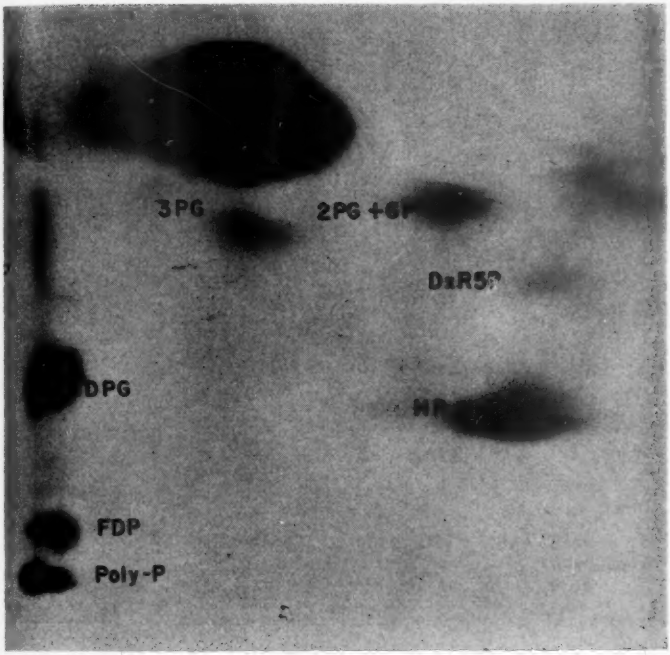


FIG. 3. Radioautogram of a two dimensional paper chromatogram of phosphate esters formed from deoxyguanosine incubations with rat erythrocyte extracts after 2 hours incubation.

TABLE 2

Formation of Acetaldehyde

	Deoxyribose Utilized (μmoles)	(per cent)	Acetaldehyde formed (μmoles)
Deoxyguanosine	1.38	30	1.5
Deoxyribose 5-phosphate	1.55	35	1.6

Incubation mixture contained 2.0 ml dialyzed rat erythrocyte extract, 4.48 μmoles of deoxyguanosine or 4.70 μmoles of deoxyribose 5-phosphate, 10 μmoles KH_2PO_4-P^{32}, 2.0 μmoles $MgCl_2$, and 20 μmoles Tris buffer (pH 7.4) in a total volume of 3.0 ml. Incubated for 2 hours at 37° C.

one to one relationship between substrate metabolized and acetaldehyde formed.

When the concentration of deoxyguanosine was varied from 2 to 6 μmoles per 2 ml. of incubation mixture, it was noted that in 2 hours the absolute amount of substrate utilized was between

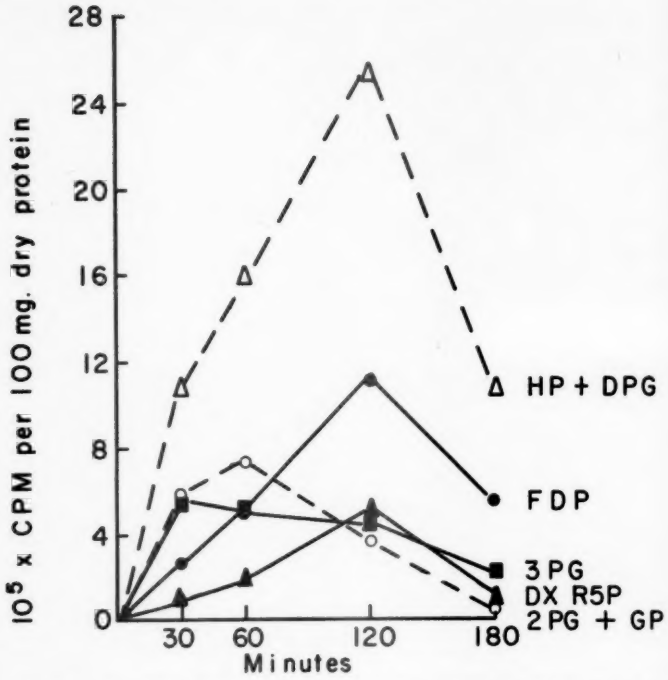


FIG. 4. Rate of individual phosphate ester formation from deoxyguanosine incubation with rat erythrocyte extracts. Phosphate esters separated by single dimension paper chromatography (see also Figure 2).

TABLE 3

Effect of Increasing Deoxyguanosine Concentration on its Utilization

Deoxyguanosine Added μmoles	Deoxypentose Metabolized from Deoxyguanosine μmoles	Deoxypentose Utilized (per cent)
2.0	1.3	65
4.0	1.2	30
5.0	1.3	26
6.0	1.5	25
12.0	0.5	4

Conditions of incubation described in the experimental section.

1.2–1.5 μmoles. At 12 μmoles there was a decrease in substrate utilization to 0.5 μmoles (Table 3). Under these experimental

conditions the enzyme system was saturated with substrate at a concentration of 1.2 to 1.5 μmoles.

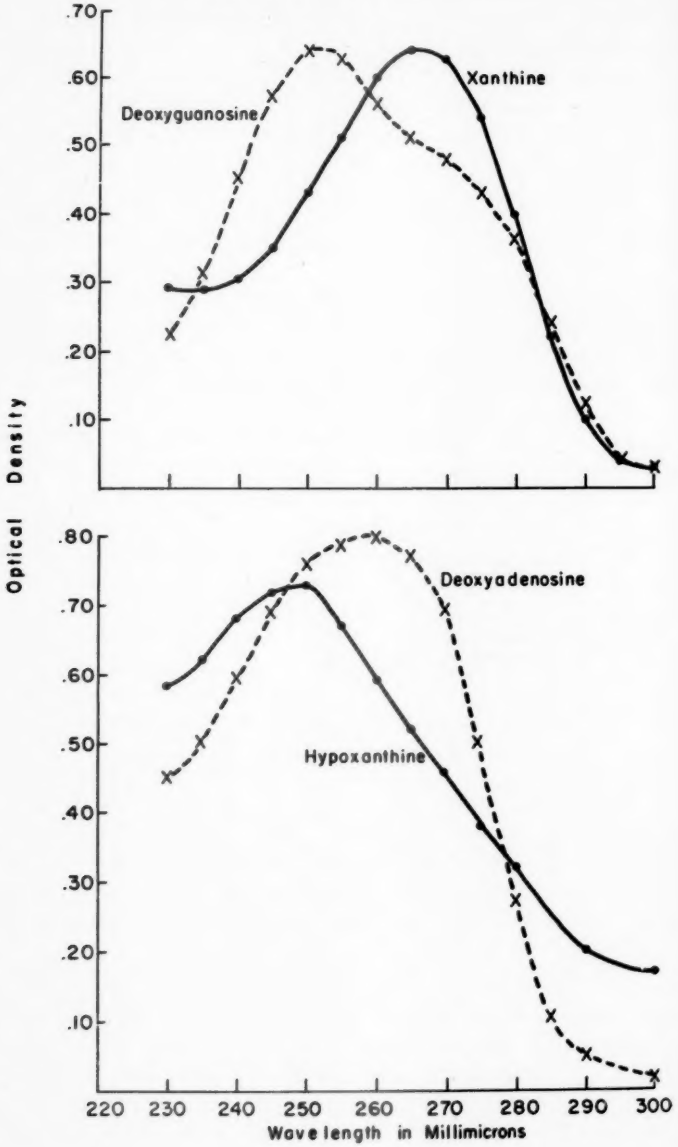
Changes in the purine moiety of deoxyguanosine and deoxyadenosine were studied following incubation with rat erythrocyte extracts. Deoxyguanosine formed xanthine, while deoxyadenosine formed hypoxanthine. These results are shown in Figure 5.

Discussion

Deoxyribonucleosides are metabolized by rat erythrocyte extracts to lactic acid and acetaldehyde (Table 1 and 2), and also to a variety of phosphate ester intermediates (Figures 1, 2 and 3). The formation of deoxyribose 5-phosphate, phosphoglyceric acids and acetaldehyde (Figures 1, 2, 3 and 4) indicates that the reaction sequence is initiated by nucleoside phosphorylase which catalyzes the formation of deoxyribose 1-phosphate and free base from the deoxyribonucleoside, as described by Friedkin and Kalckar (4). Subsequently, deoxyribose 1-phosphate is changed to deoxyribose 5-phosphate through the mediation of a deoxyribophosphomutase. The latter ester is then presumably split into 3-phosphoglycertaldehyde and acetaldehyde by the action of a deoxyriboaldolase (9). Finally triose phosphate is transformed to lactic acid. The formation of hexose phosphate is converted to hexose phosphate by aldolase. A scheme of the reactions responsible for the formation of the products identified in the dismutation of deoxyribonucleosides is summarized in Figure 6.

The purine moiety of deoxyguanosine is deaminated during incubation with rat erythrocyte extracts (Figure 5). A guanase in these extracts converts guanine into xanthine whenever the guanine is formed by the action of nucleoside phosphorylase on deoxyguanosine (Figure 6), identical to the results obtained with guanosine as the substrate (12). A deoxyadenosine deaminase is also present in these extracts (Figure 5).

Since the rat erythrocytes do not contain nucleic acids, it is very likely that the enzymes responsible for the dismutation of deoxyribosides are the remnants of the enzyme systems concerned in nucleic acid turnover in the erythroblasts from which the erythrocytes originate. These enzymes persist and make up part of the enzymic constituents of the circulating erythrocyte.


Optical Density
.70
.60
.50
.40
.30
.20
.10
Deoxyguanosine
Xanthine
.80
.70
.60
.50
.40
.30
.20
.10
Deoxyadenosine
Hypoxanthine
220 230 240 250 260 270 280 290 300
Wave length in Millimicrons

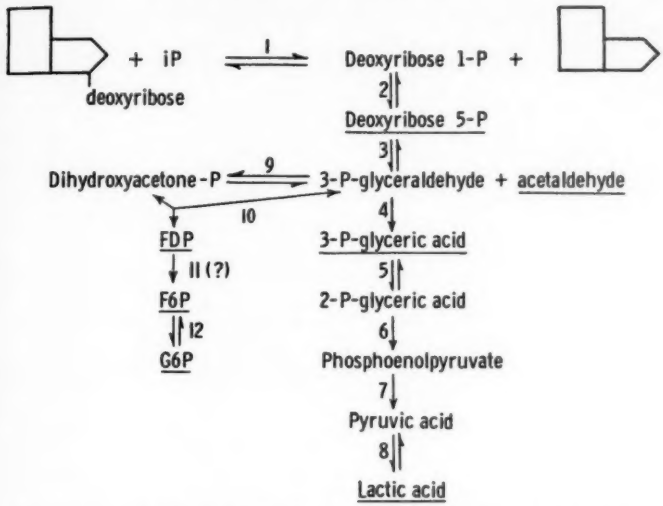


FIG. 6. A scheme depicting the formation of intermediates and products in the metabolism of purine deoxyribosides by rat erythrocyte extracts. Underlined solid lines indicate compounds that have been identified. Numbers represent the following enzymes: 1. nucleoside phosphorylase; 2. deoxyphosphoribomutase; 3. deoxyaldolase; 4. triosephosphate dehydrogenase; 5. phosphoglyceric mutase; 6. enolase; 7. pyruvic kinase; 8. lactic dehydrogenase; 9. triosephosphate isomerase; 10. aldolase; 11. fructose diphosphatase (?); and 12. phosphohexose isomerase.

Summary

1. Extracts of rat erythrocytes metabolize deoxyribose from deoxyguanosine, deoxyinosine, deoxyadenosine, and deoxyribose 5-phosphate. The products consist of a variety of phosphate esters, lactic acid, and acetaldehyde originating from the pentose moiety.

2. These products indicate that following the action of a phosphorylase on the deoxyribonucleosides, the resultant deoxyribose 1-phosphate is converted to deoxyribose 5-phosphate and the latter is subsequently split to triose phosphate and acetaldehyde. Some of the triose phosphate is then converted to hexose phosphate and the rest is further oxidized to lactic acid.

FIG. 5. Formation of xanthine from deoxyguanosine incubations and hypoxanthine from deoxyadenosine illustrated by the change in UV absorption of the unincubated controls (x–x) and the product after incubation (o–o). Studies were made on a protein free filtrate after ether extraction of TCA.

3. The base from deoxyguanosine forms xanthine and from deoxyadenosine, hypoxanthine.

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EFFECT OF PINEALECTOMY ON THE HISTOLOGY AND LIPID CONTENT OF THE RAT ADRENAL GLAND[a, b]

EDWARD G. RENNELS AND RUSSELL E. DILL[c]

Introduction

Evidence has been presented that certain extracts of mammalian pineal glands contain a substance which will increase aldosterone secretion in the dog (Farrell, 1960). The action of this agent (called adrenoglomerulotropin by Farrell since it is presumed to act on the zona glomerulosa of the adrenal cortex) is thought to be independent of the pituitary gland and higher nervous centers since its effect can be demonstrated in hypophysectomized, midcollicular-decerebrate animals.

The work of Romani *et al.* (1959) showing a stimulating action of extracts of beef pineal glands on the width of the zona glomerulosa in the rat tends to support Farrell's concept of a pineal gland hormone. On the other hand, the studies of Davis *et al.* (1961a,b) which show that an aldosterone stimulating hormone is produced by the kidney and that it can act directly to increase aldosterone output raises questions as to the physiologic importance of Farrell's "adrenoglomerulotropin."

It is, of course, well known that hypophysectomy in the rat leads to a profound atrophy of the inner cortical zones without producing a similar effect on the zona glomerulosa (Deane and Greep, 1946). It is also known that the adrenal gland of the hypophysectomized rat is still capable of producing aldosterone and there is convincing evidence that this hormone is produced by the cells of the zona glomerulosa (Turner, 1960). It seems, therefore, by analogy, that if the functional control of the zona glomerulosa is dependent in some way upon the pineal gland the removal of this gland should lead to a reduced output of aldosterone and that

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over a period of time this reduced function would be attended by morphological changes of an involutionary nature in the zona glomerulosa. Also, it would seem reasonable to anticipate that the combination of hypophysectomy and pinealectomy might produce atrophic changes in all zones of the cortex in contrast to cortical atrophy limited to the inner zones as seen after hypophysectomy. Such considerations prompted the present study which concerns the effects of pinealectomy and pinealectomy combined with hypophysectomy in the rat on the weight, histology and zonal pattern of lipid distribution in the adrenal cortex.

Material and Methods

Male rats of the Holtzman strain were used. When the animals were 25–26 days of age the operative procedures were carried out. Hypophysectomy was performed by the usual parapharyngeal approach using ether anesthesia and without tracheal cannulation. In 7 hypophysectomized animals the pineal gland was removed on the following day. This procedure was done under sodium pentabarbital anesthesia. After exposure of the calvarium overlying the pineal region a rectangular flap of bone was removed and an opening made in the dura as described by Kitay and Altschule (1954). The pineal gland was then removed by inserting the curved tips of a small pair of forceps beneath the sagittal sinus and grasping the pineal stalk. While this procedure does not permit visualization of the gland prior to its removal it obviates the necessity of transecting the sagittal or transverse sinus and causes a minimum of trauma and bleeding. Such bleeding as may occur is readily controlled by covering the exposed brain surface with a small square of gelfoam sponge. The incision is then closed with wound clips. The tissue removed was examined grossly and fixed in 10% formalin. After processing, the sectioned material was identified microscopically as pineal tissue. In addition to the 7 hypophysectomized-pinealectomized (doubly operated) animals, 7 hypophysectomized, 6 pinealectomized and 7 unoperated controls were studied.

For the duration of the experiment all hypophysectomized animals were given a 5% aqueous solution of dextrose to drink. The regular diet of laboratory chow pellets was supplemented at intervals with carrots and lettuce.

Four weeks after the operations the animals were killed by decapitation and the adrenal glands were removed, cleaned, and weighed to the nearest 0.01 mgm. on a torsion balance. The left adrenal gland from each animal was fixed in 10% formol-calcium for lipid studies while the right gland was fixed in Maximow's fluid for routine histological observations. Testes and kidneys from all animals were weighed. The pituitary glands of the intact controls and pinealectomized animals were removed and weighed. At this time a careful check of the sellar region for pituitary remnants was made in all hypophysectomized animals. Observations of hair texture (Rennels and Callahan, 1959), adrenal weights, testes weights and autopsy inspection of the sellar area indicated that all of the hypophysectomies were complete.

After fixation and subsequent infiltration overnight in 10% gelatin frozen sections were made of the left adrenal glands of all animals. Sections from the mid-region of the gland were selected and stained with Sudan Black B for total lipids. Some sections were mounted unstained for the study of birefringence and others were subjected to the Schultz test for cholesterol.

Results

As shown in Table 1, in both the hypophysectomized and doubly operated animals the usual marked suppression of body growth which follows hypophysectomy was found. It was apparent that removal of the pineal did nothing to aggravate nor to alleviate this suppression of growth. Removal of the pineal gland alone was without any significant effect on the body weight or the weight of the pituitary, kidneys or testes. A comparison of the organ weights of the two groups of hypophysectomized animals shows that the combination of hypophysectomy and pinealectomy produced no greater effect than hypophysectomy alone. Actually the adrenal gland weights were slightly greater in the doubly operated animals than in the hypophysectomized ones although this difference was not significant statistically.

Histologically, the adrenal glands can be placed into two groups; those from normal controls and from pinealectomized animals being similar and comprising one group, and those from the hypophysectomized and from the doubly operated animals forming the second group. Glands of the first group all conformed to the normal pattern of histology and lipid distribution which has already appeared in the literature many times. No specific alteration was found that could be attributed to removal of the pineal gland. The general pattern of cholesterol distribution was

TABLE 1

Effect of Hypophysectomy and Pinealectomy on Body and Organ Weights in the Rat

Animal Group	No.	Body weight gm.	Adrenals mgm.	Kidneys gm.	Testes gm.	Pituitary mgm.
Intact Controls	7	280 ± 5.08*	38.71 ± 1.38	2.37 ± 0.05	2.84 ± 0.03	9.76 ± 0.36
Pinex.†	6	277 ± 8.61	38.87 ± 1.32	2.41 ± 0.09	2.90 ± 0.06	9.52 ± 0.31
Hypex.‡	7	98 ± 3.18	9.16 ± 0.36	0.670 ± 0.03	0.134 ± 0.005	...
Hypex.+Pinex.	7	94 ± 2.80	9.94 ± 0.37	0.667 ± 0.02	0.129 ± 0.006	...

* Standard error.
† Pinealectomized.
‡ Hypophysectomized.

similar to the distribution of total lipid seen after Sudan Black staining (Figures 1 and 2). Some individual variability in amount and distribution of lipid was seen but this was largely limited to the area of the zona reticularis. Variability in this respect was as great in the glands of untreated controls as in those from the pinealectomized animals.

The second group of adrenal glands consisted of all those from hypophysectomized and doubly operated animals. All showed the histology and lipid distribution pattern characteristic of the atrophic glands of hypophysectomized rats. In such glands the cortical lipid was restricted to two zones; an outer band in the zona glomerulosa and an inner band in the zona reticularis and inner zona fasciculata. These two sudanophilic bands were separated by a rather broad sudanophobic band which corresponds to a widened "transitional" or "sudanophobic" zone and includes also the greater part of the atrophic zona fasciculata (Figures 3a and 4a). Again, the general pattern of cholesterol distribution was similar to that seen after Sudan Black B staining. The only important variation in this typical pattern of lipid distribution had to do with the degree of involvement of the inner zona fasci-

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EXPLANATION OF FIGURES

All figures are microphotographs of 10 μ frozen sections of rat adrenal glands after coloration with Sudan Black B. In each figure a strip of cortex from capsule (above) to medulla is shown. All are shown at the same magnification (X77).

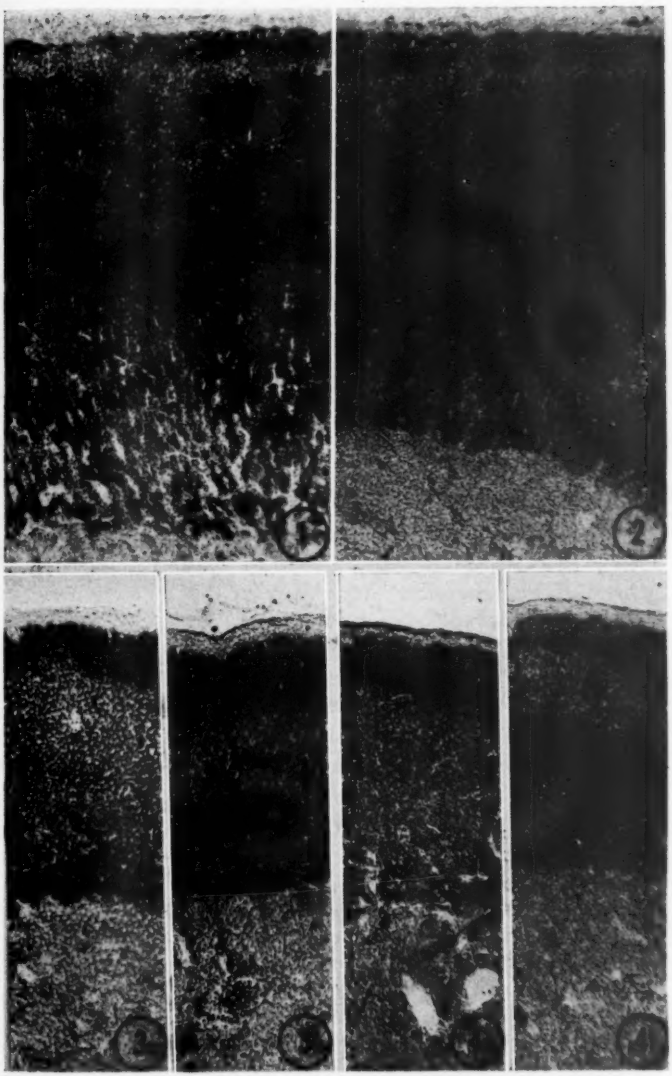
1. Adrenal gland from a normal control rat. Note the relatively narrow zona glomerulosa just below the capsule which is clearly separated from the inner zones by a clear, sudanophobic "transitional" zone.
2. Adrenal gland from a pinealectomized rat. The general pattern of lipid distribution is similar to that seen in Figure 1 except for somewhat heavier staining in the zona reticularis. Although this figure shows the cortex slightly narrower than in the normal control no consistent difference in this respect was observed. Compare the width and lipid content of the zona glomerulosa to that of the control in Figure 1.

3a. Adrenal gland from hypophysectomized rat. Note marked atrophy of inner cortical zones and reduced content of lipid. The capsule and zona glomerulosa are wider than those in the intact controls.

3b. Adrenal gland from another hypophysectomized rat showing a somewhat wider inner sudanophilic band. These two figures illustrate the range of variation in lipid pattern commonly seen in the glands of hypophysectomized rats.

4a. Adrenal gland from a hypophysectomized-pinealectomized rat. The general pattern of lipid distribution is very similar to that seen in Figure 3a.

4b. Adrenal gland from another hypophysectomized-pinealectomized rat. The pattern of lipid distribution in the cortex is quite similar to that seen in Figure 3b.



culata in lipid storage. In the adrenals of a few animals, in this second group, the inner sudanophilic band was much broader due

to lipid storage in the region of the inner zona fasciculata (Figures 3b and 4b). Since this deviation from the typical pattern was encountered in two hypophysectomized animals and also in two hypophysectomized-pinealectomized animals it can hardly be related to removal of the pineal gland. It is important to emphasize that the adrenals of the doubly operated animals differed in no way from those of the hypophysectomized controls. It is noteworthy also that no changes were observed in the zona glomerulosa that could be attributed to pineal gland removal.

Discussion

In spite of a voluminous literature dealing with the pineal gland there is still little agreement concerning its normal function (Kitay and Altschule, 1954). There is a lack of agreement concerning the effect of pinealectomy on the body weight or the weight of the adrenal glands and gonads. In the present study, pinealectomy alone caused no significant alteration in total body or organ weights.

Little attempt has been made previously to determine if pinealectomy causes histological or histochemical changes in the adrenal cortex. Farrell (1960) reported that pinealectomy in dogs did not prevent hypertrophy of the zona glomerulosa in response to sodium deprivation, although he failed to state whether pinealectomy alone produced any alteration in cortical histology. Wurtman *et al.* (1960) reported that pinealectomy of female rats failed to alter the "size and lipid content of the zona glomerulosa." Planimetric measurements of the area of the zona glomerulosa in sections were given which indicated that pinealectomy or pineal extract administration was without effect on the size of this zone but no description of the Sudan-stained preparations was presented. The combined effect of hypophysectomy and pinealectomy on adrenal histology and lipid content has not been reported previously.

In the present study, pinealectomy failed to produce any changes in the adrenal cortex that could be detected by the methods used. These methods provide information concerning the amount and distribution of total lipid (Sudan Black B) as well as of cholesterol and its esters (Schultz test and birefringence). It is now held by many investigators that these techniques provide as much useful information about the adrenal cortex as can be

obtained from a battery of so-called "ketosteroid" methods (Sayers, 1950). However, it is well known that alterations in the morphology and lipid content of the rat zona glomerulosa accompany those procedures used to decrease or increase the output of sodium retaining hormones. Thus, daily treatment with desoxycorticosterone acetate (DOCA) leads to a loss of lipid and an atrophy of the zona glomerulosa while a sodium deficient diet results in a widened zona glomerulosa and evidence of hypersecretion (Deane *et al.* 1948). If the pineal gland secretes a hormone which controls aldosterone secretion the removal of this tropic hormone should lead to a reduced secretion and associated changes indicative of hyposecretion in the zona glomerulosa. Such changes should be even more evident in the hypophysectomized-pinealectomized animals since in this case the inner cortical zones are already atrophic due to the removal of ACTH. Since, in the present study, no alterations in morphology or lipid distribution were found that could be attributed to pinealectomy it must be concluded that the presence of the pineal gland is not essential to the maintenance of the zona glomerulosa. Wurtman *et al.* (1960) have already presented evidence that pinealectomy in the rat does not interfere with aldosterone secretion.

It is possible, of course, that some area of the brain other than the pineal gland is the ultimate source of the hormone which Farrell (1959) has shown will increase aldosterone secretion in the decorticate dog. In such a case the pineal gland might serve only as a storage depot for the hormone and removal of this gland would not necessarily lead to signs of hormone deficiency. Or, it is possible that multiple regulatory systems for aldosterone secretion may exist and that adrenoglomerulotropin simply serves to modulate a basic homeostatic mechanism which can operate within certain limits without its presence. Finally, the suggestion by Farrell (1960) that, in addition to adrenoglomerulotropin, another substance which inhibits aldosterone secretion may be present in pineal gland extracts does little to clarify the problem.

It seems necessary to conclude from the results of the present study that, for the rat at least, the presence ofthe pineal gland is not essential for the maintenance of the integrity of the zona glomerulosa of the adrenal gland.

Summary

The effect of pinealectomy and pinealectomy combined with hypophysectomy on the histology and lipid content of the adrenal cortex was studied in young male rats. The animals were maintained for four weeks following the operations and at autopsy no specific effect of pineal gland removal on body weight or the weight of adrenal glands, testes, kidneys or the pituitary gland was found. Lipid studies were made on frozen sections of adrenal glands of all animals. No alterations in histology or in lipid content of the cortical cells were observed as a result of removing the pineal gland. The adrenal glands of the hypophysectomized-pinealectomized animals showed only the well-known atrophy of the inner zones which result from hypophysectomy. No indication was found that the pineal gland is essential for maintenance of the zona glomerulosa.

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QUANTITATIVE STUDIES OF REOVIRUS (ECHO 10) IN MONKEY KIDNEY CELL CULTURES[a]

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Introduction

Reovirus (ECHO 10) is one of the common infectious agents of man, yet many of its properties remain to be defined. In large measure this is because a plaque assay has not hitherto been developed for this group of viruses. Plaque titrations of infectious particles have proven to be virtually essential for quantitative studies in present day virology.

This report describes the details of a plaque test for the reoviruses, its precision, and its reproducibility. The comparative sensitivity of plaque production in kidney cell cultures of different species of monkeys has been studied. Titers obtained in plaque forming units (PFU), and in TCD_{50} by the cytopathic endpoint, are compared.

Materials and Methods

Virus. The 716 strain* (10) of type 1 reovirus (ECHO 10) was used in its 9th passage in rhesus monkey kidney (MK) cells. Stock virus was prepared in rhesus MK cells in M-E medium without serum (maintenance medium) (8) and harvested after 5 days of incubation. Cultures were frozen and thawed to disrupt residual cells and release intracellular virus. Following freezing and thawing, the cultures were harvested, centrifuged at 1600 X G for 20 minutes, pooled and stored frozen at —20° C. The same stock of virus was used for growth and morphological studies with reovirus (11, 13).

Tissue Culture. Monolayer cultures of rhesus MK cells were grown in one-ounce bottles with M-H lactalbumin hydrolysate medium (8). Phosphate-buffered saline (PBS) (2) was used for wishing cultures.

Virus Assay. After removing the growth medium and washing twice with PBS, drained monolayers of MK cells were inoculated with 0.1 ml. of appropriate dilutions of virus in maintenance medium. After an appropriate incubation period and with two further washings as described, 5 ml of the first

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* Kindly supplied by Dr. A. B. Sabin, The Children's Hospital Research Foundation, Cincinnati, Ohio.

agar overlay were added. After the agar solidified, the cultures were turned over and kept at 37° C.

The first agar overlay contained Earle's salt solution, 0.25% skim milk, 0.4% $NaHCO_3$, 0.005% phenol red, and 1.5% agar (final concentration). After 48 hours of incubation, a second overlay of 2 ml was added. The composition of the second overlay was similar to the first except that it contained neutral red to give a final concentration of 1:60,000. Plaque counts were made on the eighth day after seeding the virus.

Experimental Results

The effect of neutral red on reovirus plaque production. Preliminary experiments (12) had shown that two determining factors in the early failure to obtain ECHO 10 plaques (5) were the inhibitory effects of neutral red and of certain sera on plaque formation. The effects of neutral red were studied further as follows. Comparative titrations were carried out using different methods of overlay. Monolayers of MK cultures were inoculated with 0.1 ml of virus suspension diluted in maintenance medium to contain approximately 80–100 PFU. They were incubated at 37° C. with occasional rocking to prevent drying of cells and to improve the distribution of the virus seed. After three hours of incubation, the bottles were removed from the incubator and washed.

The bottles were then divided into two groups. One group was overlaid by the method described above under *Materials and Methods*, that is, the first agar overlay was free of neutral red, but the dye was present in the second overlay applied two days later. The second group was overlaid only once with agar medium containing neutral red.

Figure 1 shows the difference in size and numbers of reovirus plaques obtained by the two overlay methods. Not only were the plaques larger and more readily defined with the double overlay method, but there was a tenfold increase in PFU titer. A typical experiment is shown in Table 1 where plaque counts in two sets of bottles are listed.

The effect of time of adsorption. Any biological assay system for a virus is dependent upon the infective units coming in contact with and adsorbing to susceptible cells. Experiments were designed to test the time necessary for adsorption to take place. Virus was seeded in 0.1 ml volumes into different sets of drained bottle cultures. At the end of 15, 30, 60, 120, and 180 minutes,

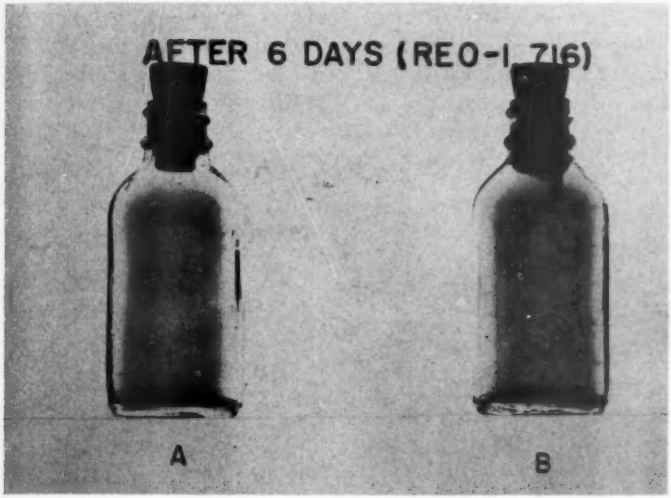

FIG. 1. The effect of neutral red on reovirus plaque production in bottle cultures, 6 days after inoculation with reovirus type 1 strain 716 (9th passage). A. Single agar overlay containing neutral red. B. Double overlay with neutral red in second overlay only.

TABLE 1

Effect of Neutral Red on the Plaque Production of Reovirus

Method	Virus dilution	No. of plaques per bottle	Average no. of plaques per bottle	Virus titer (PFU/ml)
Double overlay	10^{-5}	70, 64, 83, 92	77.4	7.7×10^7
Single overlay	10^{-5}	8, 9, 5, 8 7, 10, 9, 10	8.3	8.3×10^6

aliquot cultures were washed free of unadsorbed virus and the double overlay added. The results in Figure 2, top line, show that in the complete M-E maintenance medium, 50% adsorption occurred in about half an hour and approached a maximum at three hours.

The effect of amino acids and divalent cations. Experiments were carried out to compare the complete M-E medium (containing glucose, an enzymatic hydrolysate of a highly nutritional protein, plus the salts required for cell growth) with phosphate buffered saline with and without Ca^{++} and Mg^{++}. The reason

for determining the effect of the cation was that Puck and coworkers (9) had shown that Ca^{++} and Mg^{++} promote adsorption of bacteriophage to host cells, but the effect seems to be equivocal for enteroviruses (1, 7, 15).

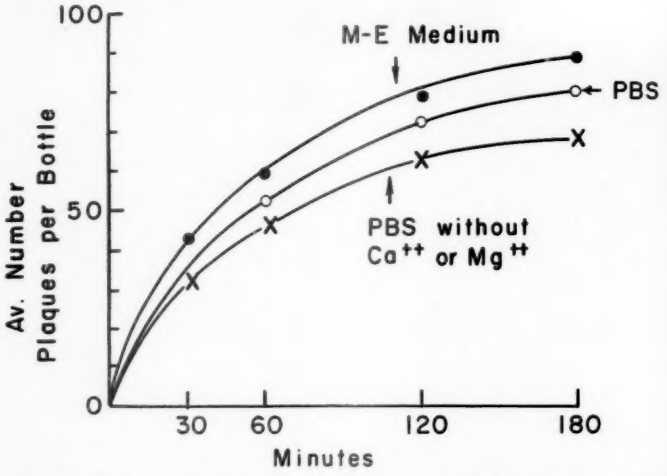

FIG. 2. Rate of adsorption of reovirus to MK cells at 37° C. from 0.1 ml volumes of each of three different diluents.

The results given in Figure 2 show that adsorption proceeded at about the same rate regardless of whether Mg and Ca were present or whether the glucose-salt solution was supplemented with the protein hydrolysate. However, the highest rate was obtained with the complete M-E medium, and its has been employed as standard in the reovirus plaque assay system.

The effect of volume of virus inoculum. The effect of volume of M-E diluent on plating efficiency was examined. The same number of PFU were placed in 0.1 ml, 0.5 ml, 1.0 ml, 2.0 ml, and 4.0 ml of maintenance medium, and these volumes were used to seed the bottle cultures. After three hours of adsorption, the fluid was removed and the double overlay added as described above. The plating efficiency was highest when the virus was present in the smallest volume and it decreased with increase in volumes inoculated (Fig. 3).

Relationship of virus concentration to plaque counts. To indi-

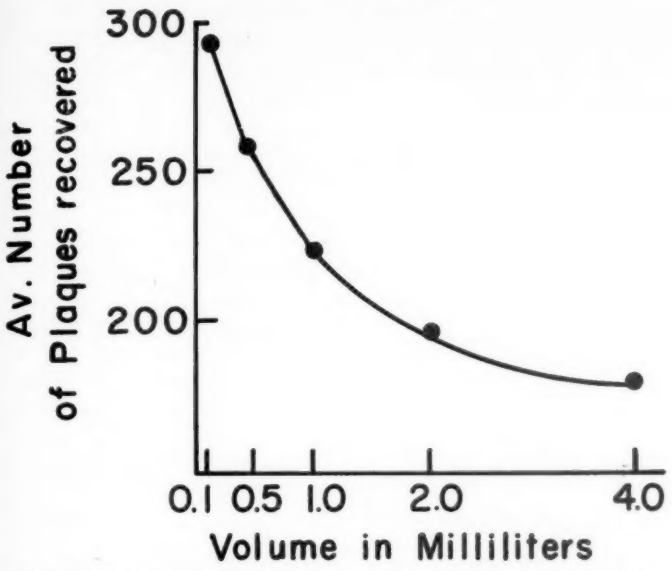

FIG. 3. Efficiency of adsorption of the same amount of reovirus diluted in different volumes of M-E maintenance medium. Time, 3 hours. Temperature, 37° C.

cate whether each plaque was produced by a single virus particle unit, the relationship of virus dilution to plaque number was determined (2). Starting at 10^{-5} concentration of virus stock, 0.1 ml of serial twofold virus dilutions was inoculated into each bottle. These were incubated at 37° C. with occasional rocking to prevent drying of cells and to improve distribution of the virus seed. After three hours, bottles were removed from the incubator, washed, and overlaid with agar medium as described above. As

TABLE 2

Proportionality Between Number of Plaques and Virus Concentration

Relative virus concentration	No. of plaques per bottle	Average no. of plaques per bottle	Virus titer (PFU/ml)
1	90, 85	88	8.8×10^7
1/2	42, 44	43	8.6×10^7
1/4	25, 21	23	9.2×10^7
1/8	17, 6	11	8.8×10^7

shown in Table 2, a linear relationship was observed between the number of plaques and the virus concentration.

Reproducibility and precision of plaque assay. The reproducibility of the plaque assay was determined by repeating the titration on the same sample on different days. It can be seen from the data in Table 3 that plaque assay by this method was highly reproducible.

TABLE 3

*Reproducibility of Plaque Assay on the Same Virus Suspension**

Experiment no.	Dilution factor	No. of plaques per bottle	Average no. of plaques per bottle	Virus titer (PFU/ml)
1	1×10^{-5}	87, 80	84	8.4×10^7
2	1×10^{-5}	85, 89	87	8.7×10^7
3	1×10^{-5}	98, 68	83	8.3×10^7
4	1×10^{-5}	83, 92	88	8.8×10^7
4	2×10^{-5}	42, 39, 48, 44	43	8.6×10^7
4	4×10^{-5}	21, 26, 19, 25	23	9.2×10^7
4	8×10^{-5}	17, 10, 6, 13	11.5	9.2×10^7
4	10×10^{-5}	9, 6, 11, 5, 7, 8, 6, 14	8.3	8.3×10^7

* The same virus stock was assayed by the plaque technique. Each numbered experiment was performed on a different day. Each culture was inoculated with 0.1 ml of virus dilution.

The sensitivity of the plaque method (PFU) compared with the cytopathic endpoint tissue culture infective doses. The same virus stock of reovirus was titrated at the same time by the plaque method and conventional tube method, using 5 tubes per tenfold

TABLE 4

Comparative Titers of Reovirus by the Plaque and the Cytopathic Endpoint Methods

Experiment no.	PFU/ml	TCD_{50}/ml	Ratio of PFU/TCD_{50}
1	7.75×10^7	10×10^7	1/1.2
2	7.85×10^7	13×10^7	1/1.6

dilution. The results are given in Table 4; ratio of PFU/ml to TCD_{50}/ml was 1/1.2 to 1/1.6.

The comparative sensitivity of reovirus plaque production in monolayers of different species of monkey kidney cells. To determine the susceptibility of reovirus plaque production, simultaneous titrations were carried out with the same virus stock in different species of MK cells. Species tested were rhesus (*Macaca mulatta*), African green (*Cercopithecus aethiops sabaeus*) and the olive baboon (*Papio doguera*) (*P. anubis*). The data in Table 5 show that rhesus cells were the most sensitive.

TABLE 5

Comparative Titers of Reovirus in Monolayers of Different Monkey Kidney Cells by Plaque Method

Monkey cells	Dilution	No. of plaques per bottle	Virus titer (PFU/ml)
Rhesus	10^{-5}	80, 70	8.0×10^7
	10^{-6}	11, 5	
Green	10^{-5}	3, 4	3.5×10^6
	10^{-6}	0, 0	
Baboon	10^{-5}	11, 11	1.1×10^7
	10^{-6}	1, 0	

Discussion

The factors involved in plaque production by reovirus (ECHO 10) were determined. The data indicate that neutral red is inhibitory to the production of reovirus plaques even with serum-free agar overlay medium (Fig. 1 and Table 1). Recently Sommerville (14) showed that failure of ECHO-2 to produce plaques was directly related to the presence of neutral red in overlay medium. Poliovirus also has been found to produce smaller and fewer plaques when the overlay medium contained neutral red and was exposed to light (4, 6, 3). The present report indicates that in order to obtain the most satisfactory assays of reovirus (PFU), we had to omit neutral red from the agar overlay medium for at least two days after virus inoculation.

The experiments on plating efficiency (Fig. 2) showed that plaques were produced in the absence of added Ca^{++} and Mg^{++} but that these cations were required for maximum virus adsorption.

In experiments on the effect of the volume of virus inoculum on plating efficiency, the data indicate that higher efficiency of plating resulted when virus was suspended in small volumes. This result was most likely due to the increased opportunity for virus to come in contact with cells before thermal inactivation occurred.

The plaque assay for reovirus described above was shown to be highly precise, reproducible and sensitive, as shown by the ratio of PFU to TCD_{50}. The validity of the plaque method for virus titrations was shown by the linear relationship between plaque number and virus concentration.

Plaque neutralization tests of normal monkey and calf sera showed that they are often inhibitory to reoviruses (12), which probably explains the failure to obtain plaques in the past when dilutions of 1:50 were used in the overlay medium (5). Using such sera in the media originally described for enteroviruses, we have confirmed the previous report on failure of reovirus plaques to appear under an overlay containing serum. In regard to screening sera for possible use in overlay media, we have found that the inhibitory titers of normal animal sera were lower (or even negligible) when tested by the conventional tube method than when tested by the plaque reduction method (12).

Summary

A technique has been developed for assaying reovirus by the plaque method in monolayers of monkey kidney (MK) cells.

The effect of some environmental variables on plating efficiency was studied. Optimal conditions included seeding the virus in a small volume of M-E medium, a three hour adsorption period, omission of neutral red from the first overlay and its addition in a second overlay 48 hours after the first. Skim milk rather than serum is recommended for the overlay. Rhesus MK cells proved more sensitive than cercopithecus and baboon cells.

Plaque neutralization tests of normal monkey and calf sera showed that they are often inhibitory to reovirus, a finding which probably explains the failure to obtain plaques in the media originally described for enteroviruses.

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ANTITUMOR ACTIVITY OF AMINO ACID ANALOGS. II. S-METHYLCARBAMOYL-L-CYSTEINE

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The introduction of reactive chemical groupings into metabolite analogs in an effort to produce noncompetitive antagonists is of considerable importance in the design of potential chemotherapeutic agents. The antitumor activity of the antibiotics, azaserine (O-diazoacetyl-L-serine) (7,13) and DON (6-diazo-5-oxo-L-norleucine) (3,4), led to a study of their effects upon the biosynthesis of purines. These antibiotics inactivate the enzyme essential for the transfer of the amido nitrogen of glutamine to formylglycinamide ribotide in the synthesis of formylglycinamidine ribotide, and the inactivation can be competitively delayed by glutamine (5). These results suggest that these analogs act by binding with the enzyme in a manner analogous to glutamine binding, but that the reactive diazo groups interact chemically with the enzyme to destroy its activity. In view of these observed antimetabolic properties of azaserine and DON, S-carbamoyl-L-cysteine was synthesized (8) in an effort to introduce a reactive chemical structure in a metabolite antagonist which would permit the analog to react irreversibly with an enzymic site after forming the enzyme-analog complex, and thus afford a mechanism for specific enzyme inactivation by appropriate antimetabolites (11). The biochemical action of S-carbamoyl-L-cysteine was subsequently studied and found to be comparable to that of azaserine in a number of biological systems (8).

The mode of action of the above antagonist may be contrasted with the so called cyto-active amino acid derivatives, e.g. *p*-di-(2-chlorethyl)aminophenylalanine (phenylalanine mustard), in which the anticipated function of the amino acid is to act as a "carrier molecule" (1). A very large number of "nitrogen mustards" of a variety of types have been synthesized (9).

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These compounds which possess chemically reactive centers are frequently toxic to the host at therapeutically active concentration levels. For example, S-carbamoyl-L-cysteine was observed to have a toxic effect on both the liver and spleen of dba mice when injected at about twice the therapeutic dosage necessary to reduce effectively the rate of tumor growth. In an effort to reduce this toxicity to the host, and yet retain the antimetabolic action of the thioester function, a number of analogs of S-carbamoyl-L-cysteine containing substituent grouping on the carbamoyl moiety were synthesized (10). A related derivative, S-methylcarbamoyl-L-cysteine, has now been found to possess appreciable anti-tumor activity with an improved therapeutic index in mice. The method of synthesis as well as a preliminary report on its anti-tumor properties are herein presented.

Experimental[c]

S-(Methylcarbamoyl)-L-cysteine

In order to obtain a maximum yield of the carbamoyl derivative, a commercial sample of L-cysteine hydrochloride monohydrate was placed over phosphorous pentoxide *in vacuo* and allowed to stand overnight. A 2.5 g. sample of the anhydrous L-cysteine hydrochloride was dissolved in 15 ml. of dimethylformamide, cooled, and 1 g. of methyl isocyanate, prepared by a previously reported procedure (2) b.p. 36–39°, was then added. After the exothermic reaction had subsided, the reaction vessel was stoppered, and allowed to stand overnight at room temperature. The resulting solution was evaporated *in vacuo* to yield a light yellow syrup which was taken up in ethanol and again evaporated to dryness. The residue was then dissolved in water, neutralized with 1 N sodium hydroxide, and a solid product was recovered by the addition of ethyl alcohol. After recrystallization from water-alcohol there was recovered 2.5 g. of material, m.p. 193–194° dec.

Anal. Calcd. for $C_5H_{10}N_2O_3S$: C, 33.69; H, 5.66; N, 15.72. Found: C, 33.97; H, 5.63; N, 15.87.

Biological Procedures

Antitumor Assays.—These experiments were carried out with an RC mammary adenocarcinoma implanted in dba mice using a procedure which was adapted in part from a previously reported technique (14). The average weight of the animals used in these experiments was about 20 g. Tumor cells grown and harvested as previously described were placed in a syringe containing a stainless steel screen of approximately 30 mesh, and twice forced through this system in order to break any large clumps of tumor. Approxi-

[c] All melting points are uncorrected. The authors are indebted to Mrs. Helene Gibson for assistance with the antitumor assays and to J. D. Glass for the elemental analyses.

mately 1 ml. of the semisolid mixture from the second step was then placed in a plain glass tissue grinding tube, an equal volume of sterile saline was added, and the mixture was macerated with a motor driven Teflon pestle. The resulting sample was then placed into sterile flasks containing about 10 ml. of saline. After mechanical dispersion, an aliquot sample was examined under a microscope for homogeneity, and a cell count was made. An appropriate volume of this latter mixture containing the desired number of cells was then implanted by subcutaneous injection near the right inguinal region. The tumor was allowed to grow for three days prior to beginning chemotherapy.

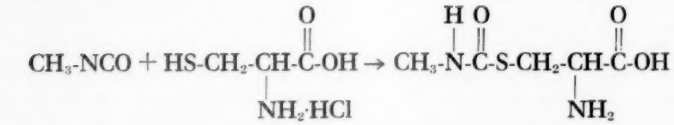
Solutions of S-methylcarbamoylcysteine decompose on standing even at neutral pH in the cold. Thus, all of the test solutions were made up from the solid just prior to injection in the animals. Each of the test animals received either of 0.2 ml. of the test solution containing 10 mg./ml. of the compound, or 0.2 ml. of sterile distilled water daily for the period of time indicated in Table 1. For each assay, the number of control animals receiving only water was equivalent to those which were injected with the test solutions.

The effectiveness of the treatment was determined by the difference in the average weight of the tumor in the experimental group as compared to the tumor growth in the control animals, and on the relative change in body weights of both sets of animals. After the animals were sacrificed to determine the effect of the treatment on tumor growth, a gross examination of the viscera was made on all of the animals in the test.

Toxicity Studies.—In addition to the terminal routine examination of the animals used in the test systems described above, a separate experiment was conducted in order to determine the lethal effects of relatively large dosages of S-methylcarbamoyl-L-cysteine. Groups of eight animals each were injected with either 0.2 ml. of the antimetabolite at the appropriate concentration or 0.2 ml. of distilled water, and after 8 days, the animals were weighed, sacrificed, and a visual examination of the internal organs was made.

Results and Discussion

S-Methylcarbamoyl-L-cysteine was synthesized in about 90% yield by the direct condensation of methyl isocyanate with anhydrous L-cysteine hydrochloride using dimethylformamide as the solvent as indicated in the accompanying equation. The parent compound S-carbamoyl-L-cysteine (8), was synthesized



in an analogous manner using potassium isocyanate, indicating that the isocyanate interaction occurs primarily at the mercapto grouping in preference to the amino radical.

The corresponding oxygen analogs of the two sulfur derivatives indicated above, O-carbamoyl-L-serine (12) and O-methylcarbamoyl-L-serine (6), had previously been prepared and found to antagonize competitively glutamine utilization in a number of microbial systems. In view of the differences in type of microbiological activities of the oxygen and sulfur unsubstituted carbamoyl derivatives, and because of the similarity of biological responses found with the O-carbamoyl- and O-methylcarbamoyl-serines, it was of considerable interest to compare the antitumor properties of the substituted and unsubstituted sulfur analogs. O-Methylcarbamoyl-DL-serine was only about one-twenty-fifth as toxic as O-carbamoyl-L-serine in several biological systems suggesting that the binding efficiency might be altered such that the toxicity to the mice of the corresponding S-methylcarbamoyl-cysteine might be less than was observed in the case of the unsubstituted thio derivative.

For each experiment, a comparable number of RC mammary adenocarcinoma cells were implanted in dba mice weighing about 20 g. each. The control animals for each experiment received the same size of tumor inoculum as did the animals to be treated, and the results are calculated based on the observed tumor growth in the control animals as compared to those receiving the chemotherapy. Under the assay conditions herein utilized, an effective dosage appears to be about 2 mg. of S-methylcarbamoyl-L-cysteine/day for about four days, or an accumulated total of about 8 mg. As indicated in Table 1, for three seperate experiments using these conditions no significant loss in body weight was observed, and the tumor growth was reduced to about 14%

TABLE 1

Antitumor Activity of S-(Methylcarbamoyl)-L-Cysteine on Mice Implanted with RC Mammary Adenocarcinoma

No. of animals used*	No. of tumor cells implanted, $\times 10^5$	Dosage, mg.	Time of experiment, days	No. of injections†	Total dosage, mg.	Biological response (per cent of control)	
						Body weight	Tumor growth
6	900	2	9	4	8	100	13
6	850	2	9	4	8	96	14
6	1300	2	9	4	8	95	14

* Average animal weight about 20 g.
† Injections given on four successive days beginning the third day after implantation.

of that observed in the control animals receiving comparable injections of a saline solution alone. Injection of only 1 mg./day of S-methylcarbamoyl-L-cysteine did not significantly affect the rate of tumor growth. A single injection of 10 mg. after the third day of implantation reduced the tumor growth to such an extent that the experimental measurement of the small weight of the tumor present in the treated animals was of doubtful accuracy; however, some weight loss was apparent in this latter treatment.

It is thus evident that S-methylcarbamoyl-L-cysteine significantly affects the rate of growth in RC mammary adenocarcinoma at a dosage of about 2 mg./day. No lethal effects were observed until about ten times this dosage was given over a two day period wherein about 75% of the animals died. In contrast, when a total of 40 mg. was injected into the animals over a four day period, all of the animals survived. Some internal damage was apparent to the spleen at these high concentration levels. In view of the antitumor activity of S-methylcarbamoyl-L-cysteine against the implanted RC mammary adenocarcinoma, additional studies concerning the toxicity and antitumor properties of this derivative are contemplated.

Summary

S-Methylcarbamoyl-L-cysteine was synthesized through the direct condensation of anhydrous L-cysteine hydrochloride with methyl isocyanate in dimethylformamide. This material is appreciably less toxic at the therapeutic dosage than the parent compound, S-carbamoyl-L-cysteine, and significantly inhibits the rate of growth of RC mammary adenocarcinoma implanted in dba mice.

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NOTE

FURTHER STUDIES ON IMMUNOLOGICAL TOLERANCE WITH MICE AND *TRICHINELLA*[a]

LEROY J. OLSON[b] AND ADAM EWERT[c]

The concept of actively acquired immunological tolerance has not been experimentally applied to infections caused by animal parasites with the exception of that reported for a helminth-vertebrate system (*Trichinella*-mice: Ewert and Olson, 1960, J. Parasitol., 46: 849–854), and with the possible exception of that reported for a protozoan-vertebrate system (*Trichomonas*-cattle: Kerr and Robertson, 1954, J. Hyg., 52: 253–263). This report presents further attempts to alter the relationship between the white mouse and a parasitic nematode, *Trichinella spiralis*.

White mice (Fairfield-Webster) were bred in the laboratory to obtain fetal or newborn animals .The stock infection of *Trichinella spiralis* was maintained in Holzman rats. Antigenic materials consisted of two types: ES antigens (larval excretions and secretions obtained by harvesting the supernate from cultured *Trichinella* larvae) and infective *Trichinella* larvae obtained by pepsin digestion of infected rat muscle (Ewert and Olson, v.s.). Intracardiac injections of newborn mice and intraperitoneal injection of fetal mice followed the methods of Grazer, 1958 (Proc. Soc. Exp. Biol. Med., 99: 407–409) and Billingham *et al.*, 1953 (Nature, 172: 603–606), respectively.

Fetal mice were given a single injection of 0.04 ml of ES antigen; approximately one-half of the fetuses in a given mouse received no injections and served as controls. Four weeks after birth, experimental and control mice were each challenged with a sublethal dose of 200 infective larvae given by stomach incubation. All mice were necropsied 13 or 30 days after challenge for the recovery of adult or larval worms, respectively. Adult recoveries from 23 ES-injected and 23 control mice averaged 14 ± 4

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and 11 ± 3, respectively; larval recoveries from 15 ES-injected and 15 control mice yielded means of 28000 ± 2500 and 30000 ± 2600, respectively.

An attempt was made to alter the response to infection of mice which at birth were given a subcutaneous injection of infective larvae. From a preliminary study it was found that larvae injected subcutaneously in the newborn mouse survived for approximately 2 days, but were not recoverable by digestion techniques on the third day from any organ of the newborn. Newborn mice were each injected subcutaneously with 500 viable larvae suspended in 0.05 ml of nutrient broth-gelatin solution; control mice (litter mates to larvae-injected mice) were similarly treated except that only the suspending solution was injected. Four weeks after birth, each mouse was challenged with 200 viable larvae by stomach intubation. All mice were necropsied 13 days after challenge for the recovery of adult worms. Adult worm recoveries from 22 larvae-injected and 22 control mice averaged 30 ± 7 and 39 ± 9 worms, respectively.

In another experiment mice were injected intracardially at birth with 0.05 ml of ES antigen, and subcutaneously each day with 0.05 ml ES antigen during a period of 3 weeks postpartum. Four weeks after birth, each mouse was given a challenge infection of 200 viable larvae by stomach intubation. Control mice (litter mates to ES-injected) were similarly treated except that only the carrier solution for ES antigen was injected. All mice were necropsied 30 days after challenge for the recovery of larvae. No significant difference was found between the mean worm burdens of 16000 ± 3100 and 22000 ± 3600 larvae for 21 ES-injected and 20 control mice, respectively.

A similar experiment was done with live larvae as the source of antigen. Experimental mice were each given a subcutaneous injection of 250 larvae at birth, and 200 to 500 larvae subcutaneously at one day intervals for a period of 3 weeks postpartum. Four weeks after birth, each mouse was challenged as described above, and necropsied 30 days after challenge for the recovery of larvae. Control mice (litter mates to larvae-injected) were similarly treated, except that only the suspending solution was injected. At necropsy it was found that 8 experimental and 10 control mice harbored averages of 5400 ± 3100 and 8400 ± 2700 larvae, respectively.

The criterion in these studies of acquired tolerance was the demonstration of a significantly greater worm burden in experimental as compared to control animals at a specified time after challenge. By this criterion acquired tolerance was not demonstrated in these experiments. Several variables may be involved in these experiments since little precedent exists for the design of this type of experiment for a helminth-vertebrate system; scheduling of tolerance-inducing injections and challenge infections, quantity and quality of materials injected, and serological responses of this host are problems not resolved by these studies. Hence, these results must be interpreted as preliminary and do not rule out the possibility of inducing tolerance in the mouse to *Trichinella*.

BOOK REVIEWS

Virus Meningo-Encephalitis. Ciba Foundation Study Group Monograph No. 7. Ed. by G. E. W. Wolstenholme and M. P. Cameron. Little, Brown & Co., Boston. 110 pp.

This small and compact book summarizes a one day conference on virus meningo-encephalitis held under the sponsorship of Ciba Foundation in London. The participants, chiefly European virologists and clinicians, reported their experiences with viruses causing meningo-encephalitis.

Macrae reviewed the different viruses causing aseptic meningitis and encephalitis in Great Britain and showed the changing pattern evolving with the reduction of poliovirus and increase of other enteroviruses as etiological agents. Similar reports, as well as clinical findings, were presented from other countries. Grist described the discovery of an apparently new ECHO prototype accounting for an aseptic meningitis outbreak in Scotland. Monkey kidney cells, usually employed for ECHO viruses, were unsatisfactory for the new Frater virus, which was most readily detected in human thyroid cultures. Recovery of enterovirus from the C.S.F. was discussed, and a report that poliovirus may be found in circulating leucocytes three to four weeks after onset of disease produced provocative comments, as warranted indeed.

Scherer pointed out that the histopathology of the Coxsackie virus can no longer be considered pathognomonic because other viruses (foot-and-mouth and certain arbor viruses) causes similar lesions in newborn mice, and also discussed experiences with encephalitis virus in Japan.

The tick-borne encephalitis complex and its epidemiology were discussed by Slonim of Prague, and Oker-Blom of Helsinki. Emphasis was given to the screening of populations by neutralization tests, and then testing the ticks for virus in areas where high percentages of immune persons may be found.

The conference was consluded by papers on the significance of inclusion bodies in meningo-encephalitis and post-infective viral encephalitis.

Eight pages of up to date references are included in the monograph. The discussions following each paper are presented. They have the advantages of spontaneity but in some instances they perpetuate comments not carefully thought out. The book is aimed for the clinician interested in virus diseases rather than for the laboratory virologist.

Asaria Ashkenazi
Joseph L. Melnick

Metabolic Effects of Adrenal Hormones. Ciba Foundation Study Group #6. Ed. G. E. W. Wolstenholme and M. O'Connor. Little, Brown and Company, Boston, 1960, VIII + 109 pp.

This is the final publication to emerge from an experimental series of six one-day study groups organized to honour the work of a member of the Ciba

Foundation's scientific advisory panel. It honours Dr. George W. Thorn, and is concerned with the metabolic effects of adrenal hormones.

The material presented is derived from the results from five laboratories, each of which is primarily interested in the effects of glucocorticoids on protein, fat and carbohydrate metabolism. There is no consideration, as might be inferred from the title, of either medullary catechol amines or cortical sex hormones.

There are two interesting chapters on the effect of glucocorticoids upon the metabolism of the liver, one each upon *in vivo* and *in vitro* effects on protein synthesis and metabolism, and one dealing with the effects on adipose tissue. Each is followed by a lively discussion on the part of several of the 27 participants, suggesting alternative hypotheses, elaborations on the material presented, or findings at variance with the thesis proposed.

Altogether, the volume of necessity limited in scope, intensively explores and lucidly presents the material covered. Although conclusions are derived from studies on experimental animals, those clinically oriented will find the extensions and restrictions of these findings as they relate to human endocrine disorders of great interest.

Charles E. Hall

Ciba Foundation Symposium on Haemopoiesis Cell Production and Its Regulation. G. E. W. Wolstenholme and Maeve O'Connor. Little, Brown and Company, Boston, 1960.

In this excellent symposium, current basic considerations of factors influencing hemopoiesis are well presented and thoroughly discussed. This volume should be of much interest to all clinical hematologists, who continue to be perplexed by problems dealing with disorders of cellular production and release to the peripheral blood, and it will occupy a particularly prominent place in the laboratories of those physiologists and hematologists who are actively engaged in this area of investigation.

The clinician may find some of the descriptions of the more sophisticated methodology to be highly specialized and not applicable to clinical studies, but every presentation in this symposium contains important conceptual discussions, all of which are pertinent to clinical problems which involve assessment of hemopoietic function. Those papers which, in their entirety, will appeal to the clinical hematologist include Lajitha and Oliver's impressive studies on the kinetics of erythropoiesis and their construction of a model of the erythron; Linman and Bethell's lucid discussion of factors controlling erythropoiesis; and Van Dyke's studies of human urinary erythropoietin.

The basic investigator will find that this volume is rich in methodology. The sophistication of radioisotope techniques employed has provided the basis for many impressive observations. Contributions by Yoffey, Everett, Cronkite and their collaborators are particularly recommended for those investigators who are active in the application of these techniques. Craddock's studies of production and distribution of granulocytes are also important contributions and will be of great interest to both basic investigators and clinical hematologists.

This Ciba Symposium is highly recommended to all who have hematologic inclinations.

William C. Levin

Ciba Foundation Symposium on Quinones in Electron Transport. Ed. by G. E. W. Wolstenholme and C. M. O'Connor, Little, Brown and Co., Boston, 1961, 453 pp., $11.00.

The collected papers and discussion of this symposium provide the first comprehensive exchange of ideas between the foremost laboratories involved in ubiquinone or coenzyme Q research. It is an exciting and rapidly expanding field, and as such the publication of this symposium is timely and will benefit workers in this field. The contributions to this volume can be divided into several categories. Four papers cover the interesting history of the isolation (Morton, Crane) and characterization of the active compound and congeners by the Wisconsin and Liverpool groups. The chemical and physical properties are discussed by the teams at Basle (Isler *et al.*) and New Jersey (Folkers *et al.*).

Six papers deal with aspects of the biosynthesis and nutrition of the quinone compounds in which the principal problems concern the mechanism of synthesis of the benzenoid nucleus. Rudney and Sugimura report that the methoxy groups of the quinone nucleus may be synthesized in animals from one carbon precursors, and Lynen *et al.* elegantly cover the biosynthesis of the terpenoid side chain. Further experiments will settle the questions of whether aromatic amino acids can serve as precursors and to what extent intestinal flora contribute to quinone levels in animal tissue. The resolution of these problems will decide whether vitamin status is to be conferred upon these compounds.

The other contributions to the symposium deal with the function of quinones in the electron transport and oxidative phosphorylation systems of animal mitochondria, microorganisms and chloroplast preparations. It now appears that the antimycin sensitive electron transport systems of most animal and plant mitochondria contain a member of the ubiquinone or coenzyme Q group. Bishop reports that plastaquinone participates in the Hill reaction of chloroplasts. The *in vivo* spectroscopic observations of ubiquinone oxidation-reduction reported by Chance are not yet published in detail elsewhere. There is an extensive discussion of the function of quinones in electron transport. Some general discussion of problems of nomenclature are also recorded in the final pages of the book, but unfortunately, no general agreement is reached by the contributors.

Lester Packer

The Nature of Sleep. Ciba Foundation Symposium edited by G. E. W. Wolstenholme and Maeve O'Connor. Little, Brown & Co., Boston. 416 pp. 101 il. $10.00.

The Ciba Symposia continue to deal with provocative subjects discussed by internationally known investigators. The present group is drawn heavily

from the British Commonwealth and the United States with representatives from France, Belgium and Switzerland. Neurophysiologists and psychologists are here but biochemists and neuroanatomists are not well represented.

The study of sleep induction and arousal is practically limited to human electroencephalographic and motor behavior and electrotactic studies in cats, intact or decerebrate at various levels. Equivalence of stages between these two is not easily determined.

The reticular core of the brain stem during sleep predominates over receptor or motor mechanisms in driving the cortex with slow periodic waves. Even in the reticular system synchronization is not dominant during waking hours when both spontaneous and driven spikes obscure any synchrony. As the sleep state is approached, receptor drives are suppressed or blocked, while the normal rhythms of the neurons (different for large and small cells) tend to synchronize. The natural periods are short for small cortical cells and become longer as we descend through striatum, thalamus to midbrain and pontine reticulum. In some states even the slowest rates from medullary levels may take precedence.

Vascular responses to activity seem to be secondary and may be very local. Mechanisms are unknown. Likewise the mechanisms for uncoupling cortical from receptor or motor drives are not known. Some feel that synaptic blocking is sufficient while others look for specific inhibiting mechanisms to account for disjunction. The old idea of accumulating metabolic depressants in the plasma is not dead. Nocturnal activity of filarial parasites may be bioassay indicators of such trace materials in plasma which the biochemists have not yet isolated.

The symposium does not come to agreed conclusions but does indicate that the data from highly specialized techniques must be correlated to obtain any general significance. The most promising advance seems to be the technique of multiple microelectrodes recorded simultaneously. This is tremendously difficult and in the lack of optical check as to exact location is subject to great errors.

John G. Sinclair

Atlas of Avian Hematology. Alfred M. Lucas and Casimir Jamroz. Agricultural monograph 25, U.S. Dept. Agr. 271 pp. 413 figures mostly in color. $4.00, Supt. of Documents, Washington 25, D.C.

The normal pattern of chicken blood is well presented in this color atlast. The blood of ducks, hawks, geese, turkey, pigeon and cuckoo is tabulated and differences are illustrated. A few of these differences are striking. Color registration is excellent and figures are large enough to present detail. Many techniques for the study of blood are critically examined. For adult blood Wright's was judged the best general stain and for developmental stages Giemsa stain modified by May-Grunwald.

The atlas is one of several volumes on chicken anatomy and histology

already partially completed by the laboratory in East Lansing. The present atlas covers other tissue cells in its marrow, spleen and thymus.

There are about 200 references corrected to 1960 and the index is very detailed. The book is a bargin for anyone interested in hematology, particularly as a basis for pathology.

John G. Sinclair

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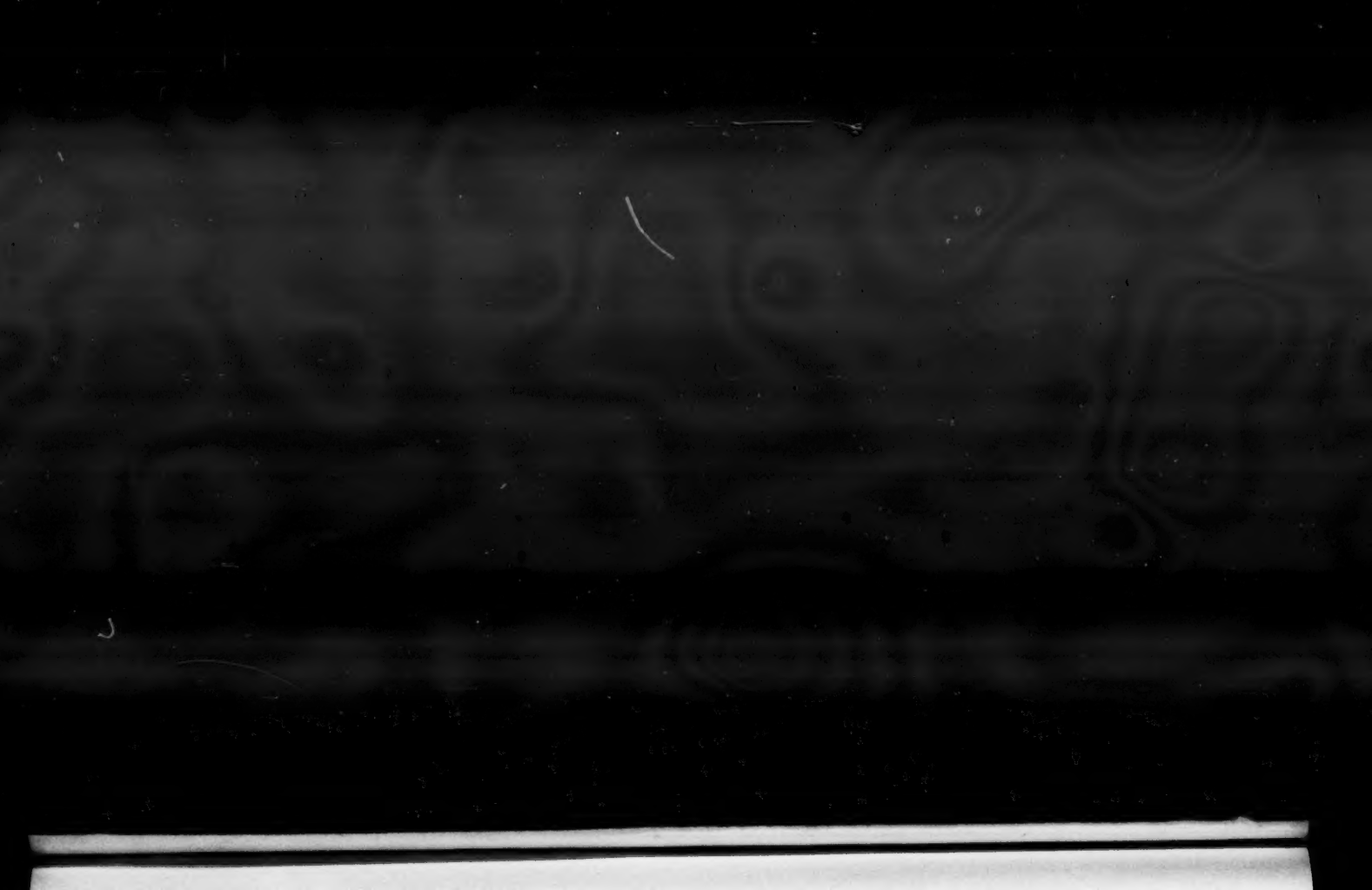
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